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(54) Title: NOVEL ENDOGLUCANASE ENZYME

(57) Abstract

The present invention concerns a novel enzyme, EGV, having endoglucanase activity. The molecular weight of the enzyme is about 20 to 25 kDA and it is isolated from the fungus _Trichoderma reesei_. The invention also relates to a DNA sequence coding for the novel enzyme as well as vectors, yeast strains and fungal strains containing the DNA sequence. Furthermore, the invention concerns a method for isolating the DNA sequence coding for the novel enzyme and for constructing yeast and fungal strains which are capable of expressing endoglucanase. The invention also provides an enzyme product having endoglucanase activity and methods for enzymatically modifying lignocellulosic materials, in particular for modification or degradation of cellulose and/or β-glucan.

NOVEL ENDOGLUCANASE ENZYME

Field of the invention

5   The present invention concerns a novel enzyme having endoglucanase activity. The enzyme is isolated from the fungus *Trichoderma reesei*. The invention also relates to an isolated and purified DNA sequence coding for the novel enzyme as well as vectors, yeast strains and fungal strains containing the DNA sequence. Furthermore, the invention concerns a method

10   for isolating the DNA sequence coding for the novel enzyme and for constructing fungal strains which are capable of expressing endoglucanase. The invention also provides an enzyme product having endoglucanase activity and methods for enzymatically modifying cellulosic/lignocellulosic materials, in particular for modification or degradation of cellulose and/or β-glucan.

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Background of the invention

Many fungal species produce enzymes that degrade plant polymers into simple compounds like sugars. The fungus *Trichoderma reesei* is one of the most potent and most studied

20   organisms degrading cellulose. It produces all the enzyme types needed for efficient break-down of crystalline cellulose, namely endo-1,4-ß-D-glucanases (EC 3.2.1.4), cellobiohydro-lases (exo-1,4-ß-D-glucanases, EC 3.2.1.91) and 1,4-ß-D-glucosidases (EC 4.3.2.21). The number of enzymes belonging to each class is far from clear, but the existence of at least two cellobiohydrolases, CBHI and CBHII, and two endoglucanases, EGI and EGII (formerly

25   EGIII,) has been confirmed by cloning of the corresponding genes (Shoemaker et al. 1983, Teeri et al. 1983, Penttilä et al. 1986, Chen et al. 1987, Teeri et al. 1987, van Arsdell et al. 1987, Saloheimo et al. 1988).

It is known in the art that the different types of cellulolytic enzymes mentioned above attack

30   different parts of the cellulose molecule, and that cellulose hydrolyzation by a cellulase mixture is the result of synergy between its components. Therefore, if total hydrolysis of a cellulose substrate is aimed at, it is generally required that the cellulase mixture contain β-glucosidases, cellobiohydrolases as well as endoglucanases. As mentioned above,

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Trichoderma reesei produces such enzyme mixtures.

It is also known that the cellulase enzymes belonging to the same class are mutually different
as regards their activities towards some of the cellulosic substrates. For example, CBHII will
5   catalyze hydrolysis of β-glucan whereas CBHI is inactive toward that substrate.

The cellulase enzymes usually consist functionally of two different parts, viz. a core and a
tail, which are interconnected by an intermittent part (known as the linker). The active centre
of the enzyme is located in the core. The function of the tail consists mainly of its capability
10   to attach the enzyme to an insoluble substrate. Thus, if the tail is removed the activity of the
enzyme toward macromolecular and crystalline substrates can be substantially decreased.

By way of a general definition, the name "endoglucanases" is assigned to enzymes that
catalyze random hydrolysis of β-1-4 glycosidic bonds between glucose units of cellulose
15   polymers. The two major *Trichoderma* endoglucanases, EGI and EGII, contain about 500 to
600 amino acids and their molecular weights are about 50 to 60 kDa. Also the cellobiohydro-
lases are similar in size. These kinds or rather bulky molecules may have difficulties in
penetrating some fibrous substrates whose adjacent polysaccharide chains are aligned and
located close to each other. Such substrates are represented by fibrous materials of great
20   economic values, such as cellulose pulp. Therefore, endoglucanases of a low molecular
weight have been of an increasing interest during the last years.

Håkansson et al. (1978) have purified a small endoglucanase from culture filtrates of
T. reesei. This enzyme has a size of about 20 kDa, a neutral pI and, unlike the major
25   cellulases, it does not contain carbohydrate moieties. Håkansson et al. found the enzyme to
be present in the culture medium in very small amounts. Small endoglucanases of similar
properties have also been isolated by Gong et al. (1979) and Ülker and Sprey (1990).
However, although the molecular weight of the endoglucanase isolated by Håkansson and
partially sequenced by Ståhlberg in 1991 is rather low, the molecular configuration of the
30   enzyme is not advantageous as far as enzymatical applications are concerned. The molecule
appears not to contain a linker domain and a cellulose binding domain (CBD) but only a core
domain. The cellulose binding domain and a linker region, allowing for its flexible separation

from the catalytic core, are essential features of true cellulases capable of efficient attachment to the substrate.

The PCT Application No. PCT/US91/07276 discloses an endoglucanase enzyme, called EGIII, derived from *Trichoderma*. The molecular size of the EGIII is 23 to 28 kDa, its pH optimum is 5.5 to 6.0 and the pI 7.2 to 8.0. From the sequence data of EGIII, it is apparent that said enzyme is the same as the one isolated by Håkansson and sequenced by Ståhlberg and that it does not contain the linker and CBD domains.

Known in the art are also small endoglucanase enzymes isolated from other microorganisms. Thus, a gene coding for a polypeptide homologous to the short amino acid sequence available from the protein described above has been isolated from the fungus *Aspergillus aculeatus* (Ooi et al. 1990). The PCT Patent Application No. PCT/DK91/00123 describes an endoglucanase derived from the fungus *Humicola insolens*. The size of the polypeptide molecule is 43 kDa and its isoelectric point is 5.1. The use of the enzyme for treatment of cellulose-containing fabrics is suggested.

Nothing has so far been reported on the existance of a small size, true *Trichoderma* endoglucanase having cellulose binding regions. It has been a general conception that the cellulase system of *Trichoderma* consists of at least two CBH:s and two EGs and additionally of the EGIII which lacks a CBD.

Isolation and manipulation of the cellulase genes is very important for the various commercial uses of enzymes and of the organisms producing them. Isolation of hydrolase genes from eukaryotes has been a task demanding either extensive studies on the corresponding enzymes or the laborous differential hybridization protocols.

Summary of the invention

It is an object of the present invention to provide a novel endoglucanase enzyme of low molecular weight and having a suitable configuration for enzymatical applications.

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This invention provides an endoglucanase enzyme derived from *Trichoderma reesei* which (in unglycosylated form) has a molecular weight of about 20 to 25 kDa and contains 242 amino acids (the mature protein contains less amino acids that that depending on the signal sequence. cleavage site), some 70 % of which are located in the core region, whereas roughly one sixth

5   of the amino acids is in the linker, taking an extended conformation, and one sixth in the CBD domain. This distribution of the amino acid residues within the molecule gives evidence of it having an elongated, "wormish" form in comparison to other cellulases, which facilitates penetration between adjacent molecules of fibrous cellulosic substrates. Being different in structure and activity, the enzyme complements the cellulolytic enzyme mixture.

10   acting in synergy, as the Examples below will show.

Another object of the invention is to provide a simple and rapid method for isolation of endoglucanase genes by function. In fact, the method described in more detail below, makes it possible to isolate any hydrolytic enzyme gene, such as genes coding for cellulases (for

15   instance endoglucanases and cellobiohydrolases) and hemicellulases (for instance xylanases and mannanases), without previous knowledge of the corresponding proteins. In this connection it should be pointed out that before this invention there did not exist any data on protein level which would have suggested the existence of the novel endoglucanase described herein. This fact is already indicative of the unusual properties resulting in its disregard in

20   the biochemical characterization of the cellulase mixture produced by *Trichoderma*.

According to the present method, an expression cDNA library is made from the organism of choice into a yeast expression vector. Yeast transformants are screened on plates containing the substrate of the desired activity. Using our earlier finding (Penttilä et al. 1987, 1988) that

25   yeast produces and secretes the major cellulases of *T. reesei* in active form, the enzymatic activities can be visualized on substrate plates.

In the following description of the present invention, the novel gene coding for the novel endoglucanase enzyme is characterized as is its transfer into, and the expression thereof, in

30   suitable hosts, such as fungi of the genus *Trichoderma*, in particular various *Trichoderma reesei* strains, and yeasts, such as *Saccharomyces cerevisiae*.

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The invention also provides yeast and fungal strains transformed with the gene coding for the novel endoglucanase enzyme. Finally, applications of the enzyme are suggested.

Brief description of the drawings

5   Fig. 1 shows the nucleotide sequence of the gene *egl5* coding for the novel enzyme, EGV.
Fig. 2A shows the cellulose binding domains and Fig. 2B linker regions of EGV compared with the same domains and regions of the other *Trichoderma* cellulases. In Fig 2B, the serine and theronine residues have been boxed.

10   Fig. 3 shows the endoglucanase gene *egl5* integrated into plasmid pAJ401 resulting in plasmid pAS4.
Fig. 4 shows the endoglucanase gene *egl5* integrated into plasmid pMLO16del5 resulting in plasmid pAS16.
Fig. 5 shows the structure of plasmid pMLO16,

15   Fig. 6 shows the structure of plasmid pMLO16del5(11),
Figs. 7a to 7d depicts the construction of the *egl5* expression plasmid pALK956, Fig. 7a also indicating the structure of plasmid pAS13,
Fig. 8 indicates the relative activity of the novel endoglucanase enzyme as a function of the pH,

20   Fig. 9 shows the pH stability of the enzyme, and
Fig. 10 shows the introns and coding sequence of the *egl5* gene.

Detailed description of the invention

25   In the following description, the following abbreviations and definitions are used:

Abbreviations:

aa, amino acid(s); bp, base pair(s); CBD, cellulose-binding domain; CBH, cellobiohydrolase;
30   *cbh*, gene coding for CBH; CMC, carboxymethyl cellulose; EG, endoglucanase; *egl*, gene coding for EG; HCA, hydrophobic cluster analysis; HEC, hydroxyethyl cellulose; kb, kilobase(s); kDa, kilo dalton(s); MUC, 4-methyl-umbelliferyl ß-D-cellobioside; MUL, 4-methyl-

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umbelliferyl ß-D-lactoside; NMR, nuclear magnetic resonance; PCR, polymerase chain
reaction; *PGK*, 3-phosphoglycerate kinase gene of *Saccharomyces cerevisiae*; pI, isoelectric
point.

5   Definitions:

Within the scope of the present invention, the term "cellulase" is used as a collective term
which encompasses enzymes catalyzing reactions which participate in the degradation of
insoluble cellulose or cellulosic substrates to soluble carbohydrate. "Cellulase" is known in
10   the art to refer to such a group of enzymes. As mentioned above, for hydrolysis of cellulose
to glucose, three cellulase enzymes (three types of cellulase enzyme activity) are needed: ran-
domly cleaving endoglucanases (1,4,-β-D-glucan glucanohydrolase, EC 3.2.1.4) which
usually attack substituted soluble substrates; cellobiohydrolase (1,4-β-D-glucan cellobiohydro-
lase, EC 3.2.1.91) which is capable of degrading crystalline cellulose but has no activity
15   towards derivatized cellulose and β-glucosidase (β-D-glucoside glycohydrolase, EC 3.2.1.21)
which degrades cellobiose and cello-oligosaccharides to yield glucose. Each of the three main
types of enzymes listed above occurs in multiple forms. For example, two immunologically
distinctive cellobiohydrolases, CBHI and CBHII are known. In addition, at least two distinct
endoglucanases are known. Synergistic action between some of these enzymes has been
20   demonstrated. "Cellulase activity" is synonymous with cellulolytic activity.

Enzymes having "endoglucanase activity" are, within the scope of the present invention,
enzymes which will catalyse the hydrolysis of internal β-1,4-linkages of cellulose.

25   By "enzyme preparation" is meant a composition containing enzymes which have been
extracted from (either partially or completely purified from) the microorganisms (for instance
the fungi) producing them. The term "enzyme preparation" is meant to include a composition
comprising medium used to culture such microorganisms and any enzymes which the micro-
organisms have secreted into such medium during the culture.

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"Culture medium" denotes a medium previously used to culture a fungi ("spent" culture
medium), such culture medium containing enzymes which the fungi have secreted into the

medium during the culture. The culture medium can be used as such or as partially or completely purified, concentrated, dried or immobilized.

5   By "hybridization" are meant conditions, under which all the different forms of DNA sequences hybridize to the DNA sequence encoding for the *Trichoderma* enzyme having endoglucanase activity, the molecular weight of the unglycosylated form of said enzyme being about 20 to 25 kDa and containing 242 amino acids (the mature protein having less amino acids).

10   "Gene" denotes a DNA sequence containing a template for a RNA polymerase. RNA that codes for a protein is termed messenger RNA (mRNA).

It is well known that mutations occur in genes and can cause changes in the amino acid sequence of the encoded polypeptide. Changes can also be introduced by genetic engineering

15   techniques. As used herein, the term *egl5* gene includes all DNA sequences homologous with the sequence herein disclosed for *egl5* and encoding polypeptides with the fuctional or structural properties of the about 20 to 25 kDa polypeptide. It is known in the art that cellulases lacking the linker and CBD regions still exhibit catalytic activity towards the β-1,4-glucosidic linkage, and thus a smaller core polypeptide is also included in the denotion of

20   *egl5*. Sequences artificially derived from this gene but still encoding a polypeptide with the desired fuctional or structural properties are also included and encompassed by the expression "functional equivalents".

A cloning vehicle or a vector is a plasmid or phage DNA or other DNA sequence (such as a

25   linear DNA) which provides an appropriate nucleic acid environment for the transfer of a gene of interest into a host cell. The cloning vehicles of the invention may be designed to replicate autonomously in prokaryotic and eukaryotic hosts. In *Trichoderma*, the cloning vehicles generally do not autonomously replicate and instead, merely provide a vehicle for the transport of the gene of interest into the *Trichoderma* host for subsequent insertion into

30   the *Trichoderma* genome. The cloning vehicle may be further characterized by one or a small number of endonuclease recognition sites at which such DNA sequences may be cut in a determinable fashion without loss of an essential biological function of the vehicle, and into

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which DNA may be spliced in order to bring about replication and cloning of such DNA. The cloning vehicle may further contain a marker suitable for use in the identification of cells transformed with the cloning vehicle. Markers, for example, are tetracycline resistance or ampicillin resistance for *E. coli* and for example phleomycin resistance or acetamidase for

5   *Trichoderma*. The word "vector" is sometimes used for "cloning vehicle." Alternatively, such markers may be provided on a cloning vehicle which is separate from that supplying the gene of interest.

A vehicle or vector similar to a cloning vehicle but which is capable of expressing a gene of

10   interest which has been cloned into it, after transformation into a desired host, is called an expression vector. In a preferred embodiment, such expression vehicle provides for an enhanced expression of a gene of interest which has been cloned into it, after transformation into a desired host.

15   The gene of interest which is provided to a fungal host as part of a cloning or expression vehicle integrates into the fungal chromosome. Sequences which derive from the cloning vehicle or expression vehicle may also be integrated with the gene of interest during the integration process.

20   The gene of interest may preferably be placed under the control of (i.e., operably linked to) certain control sequences such as promoter sequences provided by the vector (which integrate with the gene of interest). If desired, such control sequences may be provided by the fungal host's chromosome as a result of the locus of insertion.

25   A nucleic acid molecule, such as DNA, is said to be "capable of expressing" a polypeptide if it contains expression control sequences which contain transcriptional regulatory information and such sequences are "operably linked" to the nucleotide sequence which encodes the polypeptide.

Method of isolating genes

Bacterial cellulase genes have widely been isolated by transforming genomic libraries into *E. coli* and screening activities on cellulose-containing plates (reviewed by Béguin et al. 1987). This approach relies on the functionality of promoter sequences from other prokaryotes in *E. coli* and is not applicable to eukaryotes. Furthermore, eukaryotic genes, such as the *T. reesei* EGV described here, contain introns which cannot be excised in *E. coli* and thus disturb the reading frame. Moreover, the *Trichoderma* cellulases cannot generally be expressed in *E. coli* in active form even if expressed from cDNA coupled to bacterial expression signals. Traditionally fungal cellulase genes have been cloned using either differential hybridization, antibodies raised against the corresponding enzymes or hybridization with oligonucleotide probes based on the protein sequence of the enzymes (Béguin et al. 1987). All these methods are laborous and demand a lot of time and previous knowledge of the corresponding enzymes.

With the method according to the present invention, genes coding for new activities can be easily isolated without any previous knowledge of the protein. According to the invention, a fungal strain (e.g. *Trichoderma*) is cultivated on a culture medium which will induce enzyme production. Such culture medium typically contains cellulosic substrate, if endoglucanase production is aimed at. After cultivation, the mRNA of the strain is isolated and the corresponding cDNA is formed. cDNA made from the organism of interest is cloned into a yeast vector to construct an expression gene library in yeast, for instance *Saccharomyces cerevisiae*. The genes of the fungus are then expressed under any suitable promoter providing sufficient expression level, such as the yeast promoter *PGK*. The enzyme, e.g. endoglucanase, is extracellularly secreted and the colonies producing the desired enzymes, e.g. the endoglucanase, can be identified on the basis of their production of enzyme activity.

Screening can be effected with activity plate assays. Thus, according to one preferred embodiment of the present invention, the endoglucanase gene is isolated by plating the expression library onto plates containing barley β-glucan as substrate. After growth the cells are washed away and the plates are stained with congo red to reveal the hydrolysis halos. Up to 50 % of the clones giving halos may contain endoglucanase. The genes coding for

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different endoglucanases can be identified by analyzing the clones.

The expression gene library can also be constructed by using some other yeast promoter which will provide a weaker level of expression. If it is to be expected that the enzyme is
5   deleterious to the yeast, the inducible GAL1 promoter would be recommendable. It is also possible to use the endoglucanase's own promoter and, for the purpose of isolating the genes, a chromosomal gene library can, in some cases, be used. The gene library can also be constructed in a single copy plasmid. Also any other yeast strain with established trans-formation procedures can be used as a host, because their secretion capabilities are usually .
10   even higher than that of *Saccharomyces*.

In summary, the invention comprises the steps of
      -   enriching the mRNA pool of a fungal strain, e.g. *Trichoderma*, producing
          endoglucanase activity in respect of the mRNA of the endoglucanase by cultivating the
15   strain in conditions which will induce the endoglucanase production of said strain,
      -   isolating mRNA from the strain,
      -   preparing cDNA corresponding to the isolated mRNA,
      -   placing the cDNA thus obtained in a vector under the control of a suitable promoter,
      -   transforming the recombinant plasmids into a yeast strain which naturally does not
20   produce significant amounts of the endoglucanase in order to provide an expression
          library,
      -   cultivating the yeast clones thus obtained on a cultivation medium in order to express
          the expression library in the yeast,
      -   separating the yeast clones producing endoglucanase from the other yeast clones,
25   -   isolating the plasmid-DNA of said separated yeast clones, and,
      -   if desired, sequencing the DNA in order to determine the DNA sequence coding for
          the endoglucanase.

Endoglucanase V and the gene *egl5*

The gene *egl5* isolated according to the above method was sequenced according to conventional methods. The DNA sequence of *egl5* is shown in Figure 1 and also indicated in SEQ
5   ID NO. 1.

The gene *egl5* codes for a previously unknown protein of 242 amino acids, the amino acid sequence of which is depicted in SEQ ID NO. 2. Interestingly, this protein contains the two conservative domains found in all *Trichoderma* cellulases, namely the cellulose-binding
10   domain (CBD) and the linker region that connects the CBD to the catalytic core domain. The approximate regions comprising these domains are indicated in Figure 1, the linker region being the part of the sequence marked with the letter B, whereas the cellulose binding domain is marked with the letter A. The putative N-glycosylation site is marked with an asterisk. At the beginning of the protein a 17 amino acid long signal sequence (Met-Lys-Ala-
15   Thr-Leu-Val-Leu-Gly-Ser-Leu-Ile-Val-Gly-Ala-Val-Ser-Ala), which is underlined in Figure 1, can be predicted. If the signal sequence cleavage occurs at this position, the mature protein consists of 225 amino acids and has a calculated molecular weight of 22.799 KDa.

The core of the endoglucanase is separately depicted in SEQ ID NO. 3. It would appear that
20   the core of the novel endoglucanase is primarily responsible for the cellulolytic activity of the novel enzyme. Thus, it is conceived that an endoglucanase enzyme product may in principle comprise the polypeptide of the core domain only. However, the surprising enzymatic properties described below are probably attributable to a combination of the above three regions and domains, and they will therefore best be obtained if the protein comprises all
25   three parts.

It is believed that the predicted 17 aa signal peptide indicated in Figure 1 can be substituted by another suitable signal peptide possibly of a different length. Such a signal sequence should typically comprise a positively charged amino acid at the beginning followed by a
30   stretch of hydrophobic amino acids. Depending on the signal sequence cleavage site *in vivo* and the possible proteolytic processing occurring frequently in cellulases, the molecular weight of the active polypeptide may vary somewhat and the novel endoglucanase is

therefore referred to as having a molecular weight in unglycosylated form of about 20 to 25
kDa.

In its O- and N-glycosylated form the enzyme can be significantly bigger having apparent
5   molecular weights of 35 kDa or even much higher when produced in the yeast
Saccharomyces. Furthermore, cellulases frequently undergo drastic proteolytic cleavage
which removes the CBD (and linker) regions and consequently the size of EGV in fungal
culture medium can be even about 115 kDa in unglycosylated form.

10   While modelling protein conformations from first principles is not possible, the high
sequence similarity between fungal CBDs warrants the construction of a homology model
(Sali *et al.*, 1990). The feasibility of modelling side chain conformations has been
demonstrated in similar cases (Blundell *et al.*, 1988, Heiner *et al.*, 1993). Modelling of the
EGV CBD revealed some interesting differences compared to the known structure of the
15   CBHI CBD. This wedge-shaped domain seems less sharp in EGV and there are some
differences in main chain and side chain conformations and in hydrophobic properties in
areas known to be important for binding of the CBHI CBD onto the cellulose surface or for
the full activity of the CBHI enzyme against crystalline cellulose. Preliminary binding data
indicate that the EGV CBD is able to bind to cellulose.
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The protein belongs to a new family K of cellulases together with the endoglucanase B of
Pseudomonas fluorescens and the endoglucanase V of *Humicola insolens* as studied by hydro-
phobic cluster analysis by Henrissat and Bairoch (1993). This strongly suggests that EGV is
structurally different from all *Trichoderma* cellulases characterized so far. Based on this, it
25   would also appear that there are catalytic differences between the present enzyme and the
other cellulases. The fact that EGV is a true endoglucanase was confirmed by ^{1}H-NMR
spectroscopy, which showed that the internal β-1,4-linkages were hydrolysed by EGV when
barley β-glucan (a soluble glucose polymer containing β-1,4- and β-1.3-linkages) was used as
substrate.
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Thus, for instance, as evidenced by Example 11 below, the novel endoglucanase appears to
work synergetically with the known endoglucanase EGII on hydroxyethyl cellulose.

Expression of the gene _egl5_

Once the vector or DNA sequence containing the construct(s) is prepared for expression, the DNA construct(s) is introduced into an appropriate host cell by any of a variety of suitable
5   means, including transformation as described above. After the introduction of the vector, recipient cells are grown in a selective medium, which selects for the growth of transformed cells. Expression of the cloned gene sequence(s) results in the production of the desired protein, or in the production of a fragment of this protein. This expression can take place in a continuous manner in the transformed cells, or in a controlled manner.

10
Expression of the gene can be obtained in any fungus with developed transformation and expression methods.

Trichoderma is an especially useful and practical host for the synthesis of the enzyme
15   preparations of the invention because _Trichoderma_ is capable of secreting protein at large amounts, for example, concentrations as much as 40 g/L culture fluid have been reported; the homologous _Trichoderma cbh1_ promoter provides a very convenient promoter for expression of genes-of-interest because it is a strong, single copy promoter which normally directs the synthesis of up to 60 % of the secreted protein from the _Trichoderma_ host; the
20   transformation system is highly versatile and can be adapted for any gene of interest; the _Trichoderma_ host provides an "animal cell type" high mannose glycosylation pattern; and culture of _Trichoderma_ is supported by previous extensive experience in industrial scale fermentation techniques. In addition, several promoters active on glucose medium can be used, which enable the production of the enzyme essentially free from other cellulases.

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Expression of the protein in the _Trichoderma_ hosts requires the use of regulatory regions functional in such hosts. A wide variety of transcriptional and translational regulatory sequences can be employed, since _Trichoderma_ generally recognize eukaryotic host transcriptional controls, such as, for example, those of other filamentous fungi. Such control
30   regions may or may not provide an initiator methionine (AUG) codon, depending on whether the cloned sequence contains such a methionine. Such regions will, in general, include a promoter region sufficient to direct the initiation of RNA synthesis in the host cell.

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According to the invention the DNA sequence encoding EGV can be transformed into *Trichoderma* and expressed, for example under the strong *cbh1* promoter, as described in EP-A 244,234 and US 5,298,405, or other promoter functional in *Trichoderma*. The DNA sequence coding for EGV can be integrated into the general expression vector pAHM110.

5   The transformation can be done as a cotransformation using two circular plasmids, the selection marker being located in one of the plasmids and the DNA sequence encoding *egl5* in the other, or the selection marker and the DNA sequence encoding the *egl5* can be located in the same plasmid, or linear fragments can be used in the transformation. Possible selection markers are, for instance, *trpC* or *argB* from *Aspergillus nidulans* or argB or pyr4 from *T.*

10   *reesei* or *amdS* from *A. nidulans* or *trp1* from *Neurospora crassa* or phleomycine or hygromycine resistance markers from bacterial origin (EP-A 244,234, US 5,298,405, and EP-B 539,395 and Ulhoa et al., 1992, Transformation of *Trichoderma* species with dominant selectable markers, Curr. Genet 21:23-26) or other selection marker shown to function in *Trichoderma* in future (Karhunen et al. 1993, High frequency one-step gene replacement in

15   *Trichoderma reesei* I, Endoglucanase I overproduction, MGG, 241: 515-522, and Suominen et al., 1993, High frequency one-step gene replacement in *Trichoderma reesei* II, Effects of deletions of individual cellulase genes. MGG, 241: 523-530.

To construct a *Trichoderma* strain producing endoglucanase V as the main cellulolytic enzyme

20   me it is possible to construct *Trichoderma* strains that do not produce the endoglucanases I and II or all other cellulolytic enzymes: endoglucanase I and II and cellobiohydrolase I and II. The desired cellulolytic genes can be made deficient (EP-A 244,234, US 5,298,405, Karhunen et al. (1993) and Suominen et al. 1993). If genes are expressed under the *cbh1* promoter the expression is repressed by glucose and thus the strains must be grown on

25   cellulose-containing medium.

Alternatively, it is possible to construct *Trichoderma* strains expressing EGV under glucose promoter. This means that the *Trichoderma* strains expressing EGV can be grown on glucose containing medium. Possible glucose promoters are, for example, glucose derepressed *cbh1*

30   promoter of the plasmid pMLO16del5(11) (*et al.*, 1992) and the promoter of the cDNA1 gene (Nakari *et al.*, 1992) or other glucose promoters.

According to the invention, there is also provided a method for producing in fungal and yeast hosts, such as the yeast *Saccharomyces* and filamentous fungi, such as *Trichoderma*, an enzyme preparation having an endoglucanase activity stemming from an endoglucanase enzyme, the molecular weight of which (in unglycosylated form) is 20 to 25 kDa.

5   Further, if desired activities are present in more than one recombinant host, such preparations can be isolated from the appropriate hosts and combined prior to use in the method of the invention.

10   Enzyme preparations

To obtain the enzyme preparations of the invention, containing elevated levels of the EGV, the recombinant hosts described above having the desired properties (that is, hosts capable of expressing the novel endoglucanase enzyme) are cultivated under suitable conditions (cf. above), the desired enzymes are secreted from the host into the culture medium, and the enzyme preparation is recovered from said culture medium by methods known in the art.

As mentioned above, the enzyme preparation can be produced by cultivating the fungal strain in conditions where the regulatory regions directing endoglucanase expression are operating, such as on a glucose-containing medium if the yeast *PGK* or *Trichoderma* glucose promoters are used. Thus, if endoglucanase V is expressed under glucose promoter, the *Trichoderma* strains can be grown on, e.g., glucose minimal medium (Penttilä et al, 1987) or other glucose containing medium, for example Bacto-Peptone 5 g/l, Yeast extract 1 g/l, KH_2PO_4 4 g/l, $(NH_4)_2SO_4$ 4 g/l, $MgSO_4$ 0,5 g/l, $CaCl_2$ 0,5 g/l and trace element $FeSO_4.7H_2O$ 5 mg/l, $MnSO_4.H_2O$ 1,6 mg/l, $ZnSO_4.7H_2O$ 1,4 mg/l and $CoCl_2.6H_2O$ 3.7 mg/l, pH 5.0 - 6.0.

The enzyme can be produced also in other conditions, such as on Solca floc cellulose, if the *Trichoderma cbh1* promoter is used, or on a galactose-containing medium, if the yeast galactose-inducible promoter is used. The cellulose-containing cultivation medium may, for instance, comprise 6 % Solca floc cellulose (BW40, James River Corporation, Hackensack, NJ), 3 % distiller's spent grain, 0.5 % KH_2PO_4, 0.5 % $(NH4)_2SO_4$, and 0.1 % struktol as an antifoaming agent (struktol SB 2023, Schill & Seilacher, Hamburg, FRG). *Trichoderma*

strains are sensitive to glucose repression and require an inducer (cellulose, lactose or sophorose). The pH should preferably be kept at approximately pH 5 to 6 by the addition of phosphoric acid or ammonia and the temperature at 30 °C during the cultivation.

5   The enzyme preparation is recovered from the culture medium by using methods well known in the art. However, the enzyme preparations of the invention may be utilized directly from the culture medium with no further purification. If desired, such preparations may be lyophilized, immobilized or the enzymatic activity otherwise concentrated and/or stabilized for storage.

10

If desired, the expressed endoglucanase protein may be further purified in accordance with conventional conditions, such as extraction, precipitation, chromatography, affinity chromatography, electrophoresis, or the like.

15   Applications of the novel enzyme

The catalytic core of the novel enzyme is the smallest of fungal or bacterial cellulases characterized. Therefore the enzyme and the enzyme preparations according to the invention have application in the treatment of pulp and paper and in the textile industry. Furthermore,

20   the enzyme can be used in the fodder industry. The properties of the novel endoglucanase are unexpected for a endoglucanase on basis of general knowledge.

Being a β-glucanase, the novel enzyme can be used for hydrolyzation of the β-glucan of barley. As a result, the viscosity of the fodder is lowered and the nutritional value of the

25   fodder is improved.

As evidenced in Example 8, the pH optimum of the enzyme is higher than those of the other endoglucanases produced by strains of the species *Trichoderma*. This favorable pH range can be utilized in many ways. One preferred application is for removing colour from denim

30   jeans; in acidic pH, reabsorption of the colour occurs, but at neutral pH there is much less reabsorption. Another preferred embodiment comprises deinking. Normally, the pH of a slurry of water and newsprint is about 5.5 to 6.0 and therefore the novel enzyme can be used

without any need for adjustment of the pH. On the other hand, coated paper contains fillers and pigments which will raise the pH of an aqueous paper slurry formed therefrom. If the pH of the slurry is lowered by adding mineral acid, at least some of the suspended or dissolved fillers and pigments may precipitate, e.g. in the form of calcium sulphate.

5

The small size and the advantageous pH range of the novel enzyme make it possible to use it for treating recycled fibre in order to improve the technical properties thereof. The enzyme is also applicable for improving pulp drainage.

10 The invention is described in more detail with the aid of the following non-limiting examples.

In the examples, the following strains and vectors were employed: *E. coli* strains PLK-F', pBluescript SK⁻, and XL-1-Blue (Stratagene) were used as hosts for plasmids and PLK-F' as 15 a host for the cDNA library. The following plasmids were used: pAS11, pAS13, pALK487 and pALK183. The *T. reesei* strain QM9414 was used as a source of RNA for cDNA preparation and Northern analysis. *T. reesei* ALKO2221 and ALKO3524 were used as hosts for EGV expression. *S. cerevisiae* strain DBY746 (α his3 1 leu2-3 leu2-112 ura3-52 trp1-289 cyhʳ cir⁺) was used as a host for the expression library. Strain MD40-4c (α ura2 trp1 leu2-3 20 leu2-112 his3-11 his3-15) was used as a host for the plasmids pMP311, pMS3, pMP11 and pMP29 carrying *eg11*, *eg12*, *cbh1* and *cbh2* genes of *T. reesei*, respectively (Penttilä et al. 1987, 1988). The yeast expression vector pFL60 (Minet and Lacroute 1990) containing the constitutive yeast *PGK* promoter and terminator, *URA3* marker gene and the 2 micron plasmid replication origin was kindly provided by Dr. M. Minet, Centre de Génétique 25 Moléculaire, C.N.R.S., France.

Example 1

Isolation of endoglucanase gene by expression in yeast and hydrolytic properties of the
yeast

5   *T. reesei* strain QM9414 was cultivated in a 10 liter fermentor at 28 °C and pH 4.0 for 42
hours. The cultivation medium used to induce hydrolytic enzyme production contained 2 %
Solka floc cellulose, 1 % distiller's spent grain, 0.2 % Locust bean gum -galactomannan
(Serva), 0.5 % KH_2PO_4 and 0.5 % $(NH_4)_2SO_4$. After 42 hours of growth, lactose (Sigma),
Birke 150 acetylglucuronoxylan and Oat spelt arabinoxylan were added in an amount of 0.1·
10   % each and the cultivation was continued for further 24 hours.

Total RNA from the *T. reesei* strain was isolated as described by Chirgwin et al. (1979), and
the poly(A)$^+$ fraction was separated by chromatography through oligo(dT)-cellulose (BRL).
cDNA, synthesized by the ZAP-cDNA synthesis kit (Stratagene), was ligated to the *Eco*RI-
15   *Xho*I cut plasmid pAJ401. Plasmid pAJ401 was derived from plasmid pFL60 (Minet and
Lacroute 1990) by changing the two cloning sites *Eco*RI and *Xho*I between the yeast *PGK*
promoter and terminator into the reverse orientation. Transformation of *E. coli* strain PLK-F'
by electroporation (Bio-Rad) according to the manufacturer's instructions yielded a library of
3.5×10^4 independent clones. Plasmids were isolated from the pool of *E. coli* transformants
20   and transformed into *S. cerevisiae* strain DBY746 by electroporation (Bio-Rad) according to
the manufacturer's instructions. Electroporation with 7 µg of plasmid DNA yielded a library
of 8×10^4 yeast transformants.

1.2×10^5 yeast cells were plated on barley ß-glucan-containing plates to a density of 2000
25   colonies / 85 mm plate and grown at 30 °C for 3 days. Colonies were replicated and the
original plates stained with Congo Red. Unstained areas around yeast colonies indicate
hydrolysis of the substrate to oligosaccharides. Colonies showing activity were picked up
from the replica plates and purified on new activity plates. Plasmids were recovered from the
purified clones and analysed by restriction enzyme digestions. 20 clones gave a similar
30   pattern of bands which was clearly different from the earlier isolated cellulase genes of *T.
reesei*.

19

Transformation of the plasmids back to yeast confirmed that the activities were caused by the cDNA inserts. One of these plasmids, pAS4 (cf. Figure 3), was studied further. The insert in the pAS4 plasmid was named *egl5* and the corresponding protein EGV.

5   *egl5* cDNA was sequenced from both strands of the original pAS4 plasmid using the Sanger dideoxynucleotide method, T7 DNA polymerase (Pharmacia) and sequence specific primers.

The sequence obtained is shown in SEQ ID NO. 1.

10   The chromosomal *egl5* gene was isolated from a *T. reesei* cosmic library (Mäntylä, A. et al. Curr. Genet. 1992, 21 471-477) by using the *egl5* cDNA as a probe. About 6 kb *Hind*III fragment was subcloned to pBluescript SK·, resulting in plasmid pAS13 (Fig. 7a). The introns and coding sequence of *egl5* gene are shown in Figure 10 (SEQ ID NO. 11).

15   The activities of the yeast strain DBY746 carrying the pAS4 plasmid were studied by plate assays and they were compared with the activities of the yeast strains producing CBHI, CBHII, EGI and EGII.

Hydrolytic activities produced by recombinant yeast cells were detected on SD plates con-
20   taining 0.1 % barley β-glucan (β-D-1,3-1,4-glucan, viscosity 20-30 c.s.; Biocon, UK, Sherman 1991) or hydroxyethyl cellulose (HEC, Fluka, Switzerland, product 54290). After growth the plates were stained with Congo Red (Merck) as described by Penttilä *et al.* (1987) to reveal the hydrolysis halos. Xylanase activity plates containing 0.2 % of a Remazol Brilliant Blue-dyed derivative of xylan (RBB-xylan, Sigma) needed no further treatment.
25   Activities against synthetic substrates, 4-methylumbelliferyl β-D-cellobioside (MUC; Koch-Light, UK) or 4-methylumbelliferyl β-D-lactoside (MUL; Lambda Probes & Diagnostics, Austria) were detected as described by Penttilä *et al.* (1987).

The EGV protein showed a clear activity against β-glucan but the activity was lower than the
30   activities of the strains producing EGI, CBHII or EGII (Table). However, the expression levels and the secretion efficiencies of foreign proteins in yeast may vary and thus it is not possible to draw any definite conclusions concerning the level of enzyme activity. Also, the

pH on the plates is not optimal for EGV function. EGV shows some activity against
hydroxyethyl cellulose (HEC) in plate assays. No activity was detected on plate assays
towards RBB-xylan or the small synthetic substrates, methylumbelliferyl cellobioside (MUC)
or methylumbelliferyl lactoside (MUL).

5

Table 1   Hydrolytic activities of the yeast strains carrying the cellulase genes of
          Trichoderma reesei. The extent of hydrolysis of the substrate was estimated
          visually and is indicated by +.

10

	EGV	EGI	EGII	CBHI	CBHII
β-glucan	++	+++++	+++	-	++++
HEC	+	++++	++	-	-
MUL	-	+++	-	++	-
MUC	-	+++	-	+	-
RBB-xylan	-	+++	-	-	-

15

20

Example 2

**Construction of endoglucanase expression vectors with truncated fragments of the *chbl*-
promoter**

25   The vector pMLO16 (Figure 5) contains a 2.3 kb *cbhl* promoter fragment (SEQ ID 4)
     starting at 5' end from the *Eco*RI site, isolated from chromosomal gene bank of *Trichoderma
     reesei* (Teeri et al, 1983), a 3.1 kb *Bam*HI fragment of the *lacZ* gene from plasmid pAN924-
     21 (van Gorcom et al., 1985) and a 1.6 kb cbh1 terminator (SEQ ID 5) starting from 84 bp
     upstream from the translation stop codon and extending to a *Bam*HI site at the 3' end (Shoe-
30   maker et al. 1983; Teeri et al., 1983). These pieces were linked to a 2.3 kb long *Eco*RI-
     *Pvu*II region of pBR322 (Sutcliffe, J.G., 1979) generating junctions as shown in Figure 5.
     The exact in frame joint between the 2.3 kb *cbhl* promoter and the 3.1 kb *lacZ* gene was
     constructed by using an oligo depicted in Figure 5. A polylinker shown in Figure 5 was

cloned into the single internal *Xba*I site in the *chb1* promoter for the purpose of promoter deletions. A short *Sal*I linker shown in Figure 5 was cloned into the joint between the pBR322 and *cbh1* promoter fragments so that the expression cassette can be released from the vector by restriction digestion with *Sal*I and *Sph*I. Progressive unidirectional deletions

5   were introduced to the *cbh1* promoter by cutting the vector with *Kpn*I and *Xho*I and using the Erase-A-Base System (Promega, Madison, USA) accordign to manufacturer's instructions. Plasmids obtained from different deletion time points were transformed into the *E. coli* strain DH5α (BRL) by the method described in (Hanahan D, 1983) and the deletion end points were sequenced by using standard methods.

10

Example 3
Construction of vectors for expression of EGV in *Trichoderma* in glucose-containing medium

15   In order to produce EGV protein in *Trichoderma reesei* QM9414 strain essentially free of other cellulases in a medium containing glucose, the plasmid pAS16 (Fig. 4) was construc- ted. There, the *egl5* cDNA was cloned under the truncated, glucose derepressed *cbh1* promo- ter of the plasmid pMLO16del5(11), generated as explained in Example 2. The plasmid contained a 1110 bp deletion in the *cbh1* promoter beginning from the promoter internal polylinker and ending 385 bp before the translation initiation site (Fig. 5). The sequence of

20   this truncated promoter is provided as SEQ ID NO. 6. Plasmid pMLO16del5(11) was diges- ted with the restriction enzymes *Ksp*I and *Sma*I. The vector part containing the glucose- derepressed *cbh1* promoter, the *cbh1* terminator and the pBR322 sequence was blunt-ended with the Mung bean nuclease, dephosphorylated with Calf intestin alkaline phosphatase and

25   ligated to the *egl5* cDNA fragment.

The yeast expression plasmid pAS4 was digested with *Eco*RI and partially with *Xho*I to isolate the full-length *egl5* cDNA. The ends of the cDNA were filled-in with the Klenow polymerase enzyme and the fragment was ligated into the *Sma*I-cleaved vector pSP73 (Pro- mega). The resulting plasmid pAS11 was digested with *Eco*RI and *Xba*I, filled-in with the

30   Klenow polymerase and ligated to the vector part of the expression vector pMLO16del5(11).

22

Twenty micrograms of the pAS16 plasmid were digested with *Eco*RI and *Sph*I, phenol-extracted, precipitated and transformed into *Trichoderma reesei* QM9414 together with three micrograms of the plasmid p3SR2 (Hynes *et al.*, 1983) containing the acetamidase gene according to Penttilä *et al.*, (1987).

5

The promoter of the cDNA1 gene (Nakari *et al.*, 1992) was also used to direct the synthesis of the EGV protein on glucose-containing medium.

The promoter of the cDNA1 gene was cloned from the chromosomal DNA by PCR using the
10   5'primer GGT CTG AAG GAC GTG GAA TGA TGG (SEQ ID NO. 7) and the 3'primer GAT GCA TCG ATC GTC CGC GGG TTG AGA GAA GTT GTT GGA TTG ATC AAA AAG (SEQ ID NO. 8). The underlined ATCGAT in the 3'primer is a *Cla*I site and the CCGCGG a *Ksp*I site.

The *egl5* cDNA and the *cbh1* terminator were cloned as one fragment from the plasmid
15   pAS16 by PCR using the 5'primer GAG AGA CCG CGG TGA TCT TCC ATC TCG TGT CTT GCT TGT AAC (SEQ ID NO. 9) and the 3'primer ATC GTG GAT CCA TTA TTA ACA CTT CGG TGG (SEQ ID NO. 10). The underlined CCGCGG in the 5'primer is a *Ksp*I site.

Eight micrograms of both of the fragments were digested with the *Ksp*I enzyme, purified from
20   agarose gel and ligated. The ligation mixture was extracted with phenol, precipitated and used
-      instead of a plasmid in the *Trichoderma* transformation together with three micrograms of the
       p3SR2 plasmid.

The Amd⁺ transformants from the pAS16 transformation were streaked twice onto plates
25   containing acetamide (Penttilä *et al.*, 1987), and then cultivated on Potato Dextrose Agar
       plates (Difco) from which spore suspensions were made. EGV production was tested from 50
       ml shake flask cultures carried out in minimal medium according to Penttilä *et al.*, (1987)
       except that the amount of glucose was 4 %, KH_2PO_4 3 %, K_2PO_4 0.8 %, $(NH_4)_2SO_4$ 0.2 %
       and the medium was supplemented with 0.2 % peptone. Glucose was added as 15 % solution
30   when necessary to keep the level above 1 % during the whole four days of the cultivation.
       The culture supernatants of 55 transformants were analyzed for activity against barley β-glucan by the DNS-method (Zurbriggen *et al.*, 1990).

23

The spore suspensions of the three best EGV-producing clones (numbers 101, 79 and 19) were purified to single spore cultures on Potato Dextrose Agar plates. EGV production was analyzed again from these purified clones as described above. The best producing transformant 101c was analysed by Southern blotting using conventional methods and the presence of the expression casette in the genomic DNA was confirmed. Northern analysis showed that the *egl5* gene was expressed from the constructs on glucose medium.

Example 4

Construction of EGV expression plasmid pALK956

The expression plasmid pALK956 (Figs. 7d) contains:

1) *T. reesei egl5* gene fused to the *cbh1* promoter. A fragment containing the *cbh1* terminator was included after *egl5* to ensure stop in the transcription.

2) *E. coli hph* (hygromycin B phosphotransferase; Gritz and Davies, 1983) as a marker gene for transformation. The gene was expressed from the *T. reesei pki* (pyruvate kinase; Schindler *et al.*, 1993) promoter.

3) Elongated *cbh1* terminator as a flanking region to ensure stop in *pki* transcription and to target the expression cassette, together with the *cbh1* promoter fragment, to the *cbh1* locus.

The construction of pALK956 is shown in detail in Figs. 7a - 7d. For the construction, the plasmids pAS11, pAS13, pALK487 and pALK183 were used. The plasmid pAS11 contains the *egl5* cDNA (Fig. 1) and pAS13 contains the chromosomal *egl5* gene (Fig. 10). The plasmid pALK487 contains the *T. reesei cbh1* promoter (the 2.2 kb *StuI - SacII* fragment originally from the plasmid pAMH110; Nevalainen *et al.*, 1991) and *cbh1* terminator (the 0.7 kb *AvaII* fragment starting 113 bp before the stop codon of the *cbh1* gene; for the *cbh1* sequence, see Shoemaker *et al.*, 1983). The plasmid pALK183 contains *hph* gene under the control of the *pki* promoter. It was constructed from pRLM$_{ex}$30 (Mach *et al.*, 1994) by changing the *cbh2* terminator to 1.6 kb *cbh1* elongated terminator (*AvaII - Bam*HI fragment).

The exact fusion of the *egl5* gene to the *cbh1* promoter was done by PCR. The 5′-primer

contained the last 26 nucleotides of the *cbh1* promoter including the *Sac*II site and the first 18 nucleotides of the coding sequence of *egl5* (5'-CAATAGTCAA<u>CCGCGG</u>ACTGCGCATCAT-GAAGGCAACTCTGGTT; the *Sac*II site is underlined, *egl5* sequence is bolded). The 3'-primer contained 21 nucleotides of *egl5* sequence, including the *Bam*HI site about 0.7 kb from

5   the beginning of *egl5* sequence (5' -GGGCGTG<u>GGATCC</u>GTCTCTTG; the *Bam*HI site is underlined). The plasmid pAS13 was used as a template in the PCR reaction.

The 0.7 kb PCR fragment (filled in with DNA polymerase I Klenow fragment and cut with *Bam*HI), containing the exact link between the *cbh1* promoter and the *egl5* gene, was ligated·

10   to *Pvu*II - *Bam*HI digested pAS11 to obtain pALK951. The fusion and the PCR fragment were sequenced to ensure that no mistakes had occurred in the PCR amplification. Plasmid pALK955, containing the fusion of·the *egl5* to the *cbh1* promoter, was obtained by ligating *Eco*RI/Klenow - *Sac*II fragment from pALK951 between the *cbh1* promoter and terminator in the plasmid pALK487 (*Bam*HI/Klenow - *Sac*II). The *hph* marker gene (under the control of

15   the *pki* promoter) and the *cbh1* 3'-flanking region (elongated terminator) were ligated to *Stu*I cut pALK955 from pALK952 (*Xho*I - *Hind*III fragment / Klenow) to construct pALK956.

The plasmid pALK952 was constructed from pALK183 by shortening the *pki* promoter compared to the promoter used in pALK183 and pRLM$_{ex}$30 (*Not*I/partial - *Xho*I, Klenow).

20

The 7.4 kb expression cassette from pALK956 can be removed with *Not*I digestion.

Thus, in summary, in the expression plasmid pALK956, the *egl5* gene is fused to the *cbh1* promoter. The *E. coli hph* (hygromycin B phosphotransferase) gene is used as a marker for

25   the transformations. The *cbh1* 3'-flanking region (elongated terminator) is included to ensure stop in the *pki* transcription and to target the expression cassette, together with the promoter fragment, to the *cbh1* locus.

Example 5

30   **Expression of EGV under the *cbh1* promoter in cellulase-inducing medium**

The EGV expression plasmid, pALK956, was digested with *Not*I, and the 7.4 kb fragment

25

was purified from agarose gel. 2-3 µg of the linear fragment was transformed into *T. reesei* strains ALKO2221 and ALKO3524 according to Penttilä et al. (1987) with the modifications described in Karhunen et al. (1993). ALKO2221 is a low protease mutant from *T. reesei* VTT-D-79125 (Bailey and Nevalainen, 1981), prepared in our laboratory (A. Mäntylä).

5 ALKO3524 is a strain derived from VTT-D-79125, where the *cbh2*, *egl2* and *egl1* genes have been deleted using the *A. nidulans trpC* (Yelton et al., 1984), *A. nidulans amdS* (Kelly and Hynes, 1985) and *Streptoalloteichus hindustanus phleo'* (Mattern et al., 1987) marker genes, respectively. The method of one-step gene replacement with a linear fragment and flanking regions of the corresponding cellulase locus is described in Suominen et al. (1993).

10

HygB+ transformants were selected on plates containing *T. reesei* minimal medium (Penttilä et al., 1987) with 100 µg hygromycin/ml. Transformants were purified by single spore selection on selective medium and then cultivated on Potato Dextrose Agar. Purified transformants were grown in shake flasks in a medium containing 4 % whey, 1.5 % complex

15 nitrogen source derived from grain, 1.5 % KH_2PO_4 and 0.5 % $(NH_4)_2SO_4$. Cultures were maintained at 30 °C and 250 rpm for 7 days. The culture supernatants were analyzed for activity against barley β-glucan at pH 6.3 by the DNS-method (Zurbriggen et al., 1990). Soluble protein was assayed by the method of Lowry et al. (1951) using bovine serum albumin as standard. The detection of the 67 kDa CBHI protein was done in SDS-PAGE

20 followed by Coomassie Brilliant Blue staining. The results from the best EGV transformants and the corresponding host strains are shown in Table 2. In the EGV-transformants the β-glucanase activity measured at the optimum pH of EGV was enhanced about twofold.

26

Table 2. Expression of EGV under the *cbhl* promoter in cellulase-inducing medium

Strain	Protein (mg/ml)	β-glucanase activity (BU/ml)	CBHI (+/-)
ALKO2221	7.1	1945	+
EGV/ALKO2221/11	5.2	3262	+
EGV/ALKO2221/47	5.5	3236	-
EGV/ALKO2221/31	3.5	3757	-
EGV/ALKO2221/68	3.9	3479	+
ALKO3524	9.3	3338	+
EGV/ALKO3524/27	5.3	6770	+
EGV/ALKO3524/28	7.6	7588	+
EGV/ALKO3524/31	7.0	6622	+

Example 6.

Enzyme preparation containing EGV protein obtained from yeast where egl5 gene was expressed.

Saccharomyces cerevisiae DBY 746 containing the pAS4 plasmid was grown in a bioreactor (Chemap LF 20, working volume 16 l) on a standard YPD medium. The inoculum (5 times 200 ml) was grown in shake flasks in selective synthetic complete medium without uracil. Cultivation conditions were: temperature 30 °C, pH controlled between 5.2 and 5.9, aeration about 15 l min^{-1} and cultivation time 45 h. The yeast cells were separated from the medium by centrifugation and the culture supernatant was concentrated 4-fold by ultrafiltration (PCI ES 625 membranes).

The enzymatic activity in the concentrate was assayed by standard methods using appropriate incubation times for the enzyme reaction against β-glucan (Zurbriggen et al., 1990a) and hydroxyethyl cellulose, HEC (IUPAC, 1987). The β-glucanase activity was 0.7 nkat ml^{-1} and

endoglucanase (HEC) activity less than 0.4 nkat ml^{-1}. No endoglucanase activity could be detected in culture filtrates of control cultivations of yeast missing the EG V gene (*S. cerevisiae* DBY 746 carrying the plasmid pAJ 401).

5   The purified EGV preparation can be obtained from the ultrafiltration concentrate by standard protein chromatography methods. The EGV protein can be bound to an anion exchanger resin (e.g. Mono Q columns or DEAE Sepharose FF, Pharmacia) in low ionic strength buffer and at appropriate pH. The protein can be eluted out of the column using increasing gradient of NaCl (e.g. from 0 to 0.5 M in the buffer of binding). Alternatively, the impurities from the ·

10   preparation of EGV can be removed by binding them in anion exchange resin at appropriate pH and ionic strength where EGV is not bound to the resin. Cation exchanger resins (e.g. Mono S columns or CM Sepharose FF, Pharmacia) can be used in analogous way by selecting buffers of appropriately low pH (e.g. pH 4 - pH 6). EGV can also be purified by gel permeation chromatography where it can be separated due to its small molecular size. The

15   columns of various materials (e.g. Sephacryl S-100 HR or various types of Sepharose and Superose, Pharmacia; Fractogel TSK HW-55, Merck) in e.g. phosphate or acetate buffers containing e.g. 0.05 - 0.5 M NaCl can be used. Hydrophobic interaction chromatography and various affinity chromatography methods may also be used.

20

Example 7
Enzyme preparation containing EG V protein obtained from *Trichoderma reesei* grown on glucose.

25   One of the best producing *T. reesei* QM9414 transformants (number QM/101c) was grown in a bioreactor (Chemap LF 20, working volume 16 l) on a medium of Mandels and Weber (1969) where Solka floc cellulose (10 g l^{-1}) was replaced by 20 g l^{-1} of glucose and where the concentrations of other nutrients were correspondively doubled. The inoculum (5 times 200 ml) was grown in shake flasks in a medium containing 40 g l^{-1} glucose and the adequate

30   mineral salts for nutrients and buffering of the medium. Cultivation conditions were: temperature 29 °C, pH controlled between 4.0 and 5.0, aeration about 15 l min^{-1} and cultivation time 93 h. During the cultivation glucose concentration in the fermentor was maintained above 5 g

l⁻¹ by adding continuously sterile glucose (40 g l⁻¹) solution. The mycelium was separated from the medium by centrifugation and the culture supernatant was concentrated 1.6 times by ultrafiltration (PCI ES 625 membranes).

5   The clarified supernatant was first fractionated by hydrophobic interaction chromatography. The pH of the sample was was adjusted to pH 6.0 and conductivity of the sample to the value corresponding to 10 mM sodium phosphate buffer, pH 6.0, containing 1.25 mol l⁻¹ $(NH_4)_2SO_4$. The sample was applied to a column (113 x 110 mm) of Phenyl Sepharose FF (Pharmacia), previously equilibrated with 10 mM sodium phosphate buffer, pH 6.0, containing 1.25 ·

10   mol l⁻¹ $(NH_4)_2SO_4$. Elution was started by the equilibrating buffer followed by a linear decreasing gradient of ammonium sulphate from 1.25 M to 0 M. Fractions (each 450 ml) which contained the major endoglucanase activity were combined, eluted at the end of the decreasing gradient and by 10 mM phosphate buffer. The other adsorbed proteins were eluted by distilled water and the column was washed with 6 M urea.

15

The enzyme preparation obtained in the first chromatographic step was equilibrated to 4 mM sodium phosphate, pH 7.2 by gel filtration (Sephadex G-25 coarse). The equilibrated protein solution was applied to a column (113 x 190 mm) of DEAE Sepharose FF (Pharmacia), pre-equilibrated with the same buffer. Elution was performed first with the equilibrating buffer to

20   remove unadsorbed proteins and thereafter by stepwise additions of sodium chloride to concentration of 200 mM. Fractions (each 900 ml) which contained the endoglucanase and which eluted by 200 mM NaCl were collected and the fraction with the highest activity was concentrated by ultrafiltration (Amicon PM-10 membranes). The specific activity of the preparate was 360 nkat mg⁻¹ protein and purification factor of ca. 30 was obtained when

25   compared to the supernatant of the fermentor liquid.

The preparate was further characterized by isoelectric focusing on PBE-94 anion exchange material (Pharmacia). The column was equilibrated by 25 mM imidazole-HCl buffer, pH 7.4 and elution was carried out by Polybuffer 74 (Pharmacia) - HCl buffer, pH 4.0 according to

30   the manufacturer's instructions. EGV, measured by β-glucanase activity, eluted from the column at pH 6.6 - 7.2.

Example 8

pH-Optimum of EGV

1.0 % solutions of barley β-glucan (Megazyme, Australia) was prepared in McIlvaine buffers,
diluted one to four (corresponding ca. 50 mM citrate-phosphate buffer), in pH range from 3.0
to 8.0. Activity of an enzyme sample prepared as described in Example 7 was assayed using
as substrate the β-glucan solutions prepared in the varying pH-values. The assay procedure
was otherwise similar to the procedure of endoglucanase assay (IUPAC, 1987). Incubation
time in the assay was 10 min at 50 °C, after which the enzyme reaction was terminated by
boiling. Reducing sugar groups formed in the reaction were measured by DNS-reaction.

The pH-optimum of EGV was 6.0 - 6.5 (Figure 8).

Example 9

pH-Stability of EGV

An EGV sample was prepared as described in Example 7, except that the last concentration
by ultrafiltration was omitted (activity 48 nkat/ml, assayed at pH 6.3 against barley β-glucan,
analogously to endoglucanase assay, IUPAC, 1987). This sample was diluted (1 part per 2
parts of buffer) by 100 mM buffers of sodium acetate and sodium phosphate, prepared in
different pH values. The diluted samples were incubated at 40 °C for 20 h, and the activity
was assayed as described earlier. The pH of incubation was measured after the incubation.

More than 80 % of the original activity was observed in the samples incubated at pH range
from ca. pH 5.4 to ca. pH 6.8. The relative recovered activity is presented in Figure 9.

Example 10

Hydrolysis of insoluble cellulosic substrates by EGV.

Avicel (Serva 14204) which is mainly crystalline cellulose and phosphoric acid-swollen

amorphous (Walseth, 1952) cellulose were hydrolysed by the new endoglucanase enzyme (EGV) alone and in combinations with the previously known cellobiohydrolases of *T. reesei*. The preparation of EGV was obtained as described in Example 7. CBHI and CBHII were purified from culture filtrates of *Trichoderma reesei* and were pure proteins as judged by

5   SDS-PAGE. As a control, the cellobiohydrolases were incubated without the addition of EGV. The reducing sugars liberated in the treatments were assayed using the DNS method and the reaction products were analysed by HPLC.

The substrates for the hydrolysis were prepared in 50 mM sodium citrate buffer, pH 5.8 in

10   concentration of 10 g l^{-1}. EGV was dosed on the basis of activity against β-glucan at pH 5.8 (500 or 2000 nkat g^{-1} substrate) and cellobiohydrolases (CBHI and CBHII) on the basis of protein (1.0 or 4 mg g^{-1} substrate). The reaction mixtures were incubated for 20 h at pH 5.8 at 40 °C after which the hydrolysis was terminated by boiling. The values for reducing sugars as glucose assayed from the reaction mixture are presented in Tables 3 and 4. The enzyme

15   dosage was 500 nkat g^{-1} substrate for EGV and 1.0 g g^{-1} substrate for CBHI and CBHII (Table 3), and 2000 nkat g^{-1} substrate for EGV and 4.0 g g^{-1} substrate for CBHI and CBHII (Table 4). Duration of the hydrolysis was 20 h in both cases.

The major hydrolysis product of EGV was cellobiose but also cellotetraose was detected in

20   the hydrolysate by HPLC. The strong synergy of EGV with CBHI in the hydrolysis of these substrates can be clearly seen. Even though EGV released only small amounts of soluble sugars the enhancing effect on the cellulose hydrolysis by CBHI was remarkable.

25   **Table 3.** Reducing sugars liberated by cellulases from *T. reesei* in the hydrolysis of crystalline cellulose (Avicel) and amorphous (Walseth) cellulose

enzymes	reducing sugars as glucose (mg ml^{-1})	
	Avicel	Walseth
EGV alone	0.00	0.01 (*)
CBHI alone	0.09	0.16

CBHII alone	0.23	0.67
CBHI and EGV	0.15	0.25
CBHII and EGV	0.23	0.74

(*) determined by HPLC

Table 4. Reducing sugars liberated by cellulases from *T. reesei* in the hydrolysis of crystalline cellulose (Avicel) and amorphous (Walseth) cellulose

enzymes	reducing sugars as glucose (mg ml^{-1})	
	Avicel	Walseth
EGV alone	0.03 (*)	0.05
CBHI alone	0.35	0.49
CBHII alone	0.43	1.44
CBHI and EGV	0.43	0.71
CBHII and EGV	0.47	1.67
EGV (larger dosage (**)	0.06	0.13

(*) determined by HPLC
(**) dosed activity 5000 nkat g^{-1} substrate

Example 11

Hydrolysis of soluble cellulosic substrates by EGV

HEC (hydroxyethyl cellulose, Fluka 54290) which is a soluble substituted cellulose polymer and barley β-glucan (Megazyme, Australia) were hydrolysed by the new endoglucanase enzyme (EGV) alone and in combinations with two previously known endoglucanases of *T. reesei*. The preparation of EGV was obtained as described in Example 7. Endoglucanases EGI and EGII were purified from culture filtrates of *Trichoderma reesei* and were pure proteins as

32

judged by SDS-PAGE. As a control, the endoglucanases were incubated without the addition of EGV. The reducing sugars liberated in the treatments were assayed using the DNS method.

5   The substrates for the hydrolysis were prepared in 50 mM sodium acetate buffer, pH 5.8 in concentration of 10 g l^{-1}. Endoglucanases were dosed on the basis of activity against β-glucan (dosage for each: 100 nkat g^{-1} substrate) The reaction mixtures were incubated at 40 °C after which the hydrolysis was terminated by boiling. The values for reducing sugars as glucose assayed from the reaction mixture for HEC are presented in Table 5, and for β-glucan in Table 6. The enhancing effect of EGV on the hydrolysis of HEC and especially β-glucan can
10   clearly be seen.

Table 5.   Reducing sugars liberated by endoglucanases from *T. reesei* in the hydrolysis of hydroxyethyl cellulose.

enzymes	hydrolysis products as reducing sugars (mg ml^{-1})	
	2 h hydrolysis	20 h hydrolysis
EGV alone	0.00	0.00
EGI alone	0.14	0.26
EGII alone	0.18	0.29
EGI and EGV	0.16	0.27
EGII and EGV	0.24	0.29

Table 6. Reducing sugars liberated by endoglucanases from *T. reesei* in the hydrolysis of barley β-glucan. Duration of hydrolysis 2 h.

enzymes	hydrolysis products as reducing sugars (mg ml^{-1})
EGV alone	0.96
EGI alone	1.1
EGII alone	0.90
EGI and EGV	2.4
EGII and EGV	2.0

Example 12

Modelling of the cellulose-binding domain of EGV

Sequences of the five cellulose-binding domains (CBD) of the *T. reesei* cellulases were aligned to define conserved regions using the computer program MALIGN (Johnson *et al.*, 1993; Johnson and Overington, 1993), which is suitable for this purpose because the percentage identity between the five CBDs is ~60 %. The construction of a 3-D model of the EGV CBD was performed using the COMPOSER method (Sutcliffe *et al.*, 1987a,b; Blundell *et al.*, 1988; Sali *et al.*, 1990), which is based on rules derived from known three-dimensional structures. These rules can be used to define a conserved core for the model, to select appropriate fragments for the variable regions and to replace the side chains. The NMR-based structure of the CBHI CBD (Kraulis *et al.*, 1989) was used as a basis for the EGV model. The computer program CHARMm ver. 22 (Brooks *et al.*, 1983) was used to soak the completed model in a 35 Å cubic box of water and to refine the model through energy minimization and molecular dynamics simulation of 100 ps under periodic boundary conditions.

The sequence alignment shows that the CBDs of *T. reesei* are highly conserved except for one insertion and one deletion of a single aa in EGV. Therefore most parts of the 3-D structure of the CBHI CBD, determined by NMR (Kraulis *et al.*, 1989), could be used as a conserved core

34

for modelling of the EGV CBD by the computer program COMPOSER. The CBHI CBD is a wedge-shaped domain having two flat surfaces. One of these is predominantly hydrophilic and contains three tyrosine residues that have been shown by chemical modification to be important for the binding of the enzyme to cellulose (Claeyssens and Tomme, 1989). Tyr^{492} located

5   at the tip of the wedge has also been demonstrated by site-directed mutagenesis to be involved in substrate binding (Reinikainen et al., 1992). This residue is replaced by a tryptophan (Trp^{236}) in the EGV CBD (Fig 3B), an amino acid substitution also seen in many other fungal CBDs. Both tyrosine and tryptophan residues interact readily with carbohydrates.

10   The backbones of the CBHI and EGV CBDs are very similar. Two disulfide bridges in identical positions stabilize the structures. The insertion and the deletion in EGV are situated in a single loop and thus compensate each other, maintaining the loop backbone unchanged compared with that of CBHI. However, there is an interesting difference in the backbone conformation at the other, more hydrophobic, flat face. A substantial change in torsion angle

15   was observed at position Gly^{220} of EGV, where the ϕ-angle of the glycine residue changes from negative to positive during the refinement simulation. This causes the loop at region 217-221 to be pushed outwards. Interestingly, the corresponding loop in the CBHI CBD contains a proline residue (Pro^{474}), mutation of which reduces the activity of CBHI against crystalline cellulose (Reinikainen et al., 1992). Simulations of the other CBDs from *T. reesei*

20   show that this region is the most flexible region of the CBD (A.-M. Hoffrén, T.T. Teeri, and O. Teleman, submitted).

There are also moderate differences in the backbone regions, in which proline (Pro^{491}) and serine (Ser^{482}) residues of the CBHI CBD are replaced by glutamine (Gln^{235}) and proline

25   (Pro^{227}) residues in the EGV CBD, respectively. Moderate differences are also found in side chain conformations, but these changes are within the range of fluctuation occurring during simulation. One of the tyrosines (Tyr^{210}) forming the hydrophilic face of EGV CBD points more upwards than its counterpart (Tyr^{466}) in CBHI. This difference in orientation is significant, but the flexibility allows Tyr^{210} in EGV to occupy the same position as Tyr^{466} in CBHI.

30   Thus the difference in orientation is unlikely to affect substantially the affinity for cellulose. Overall, the EGV CBD seems to be less wedge-shaped and the hydrophobic surface more rounded than that of the CBHI CBD.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

5

    (i) APPLICANT:
        (A) NAME: OY ALKO AB
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        (C) CITY: Helsinki
10   (E) COUNTRY: Finland
        (F) POSTAL CODE: FIN-00180

    (ii) TITLE OF INVENTION: Novel Endoglucanase Enzyme

15   (iii) NUMBER OF SEQUENCES: 10

    (iv) COMPUTER READABLE FORM
        (A) MEDIUM TYPE: Floppy disc
        (B) COMPUTER: IBM PC compatible
20   (C) OPERATING SYSTEM: PC-DOS/MS-DOS
        (D) SOFTWARE: PatentIn Releae #1.0, Version #1.25 (EPO)

    (v) PRIOR APPLICATION DATA:
        (A) APPLICATION NUMBER: FI 932521
25   (B) FILING DATE: 2-JUNE-1993

(2) INFORMATION FOR SEQ ID NO: 1:

30   (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 884 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: double
        (D) TOPOLOGY: linear
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    (ii) MOLECULE TYPE: cDNA

    (iii) HYPOTHETICAL: NO

40   (iii) ANTI-SENSE: NO

    (vi) ORIGINAL SOURCE:
        (A) ORGANISM: Trichoderma reesei
        (B) STRAIN: QM9414
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    (ix) FEATURE:
        (A) NAME/KEY: CDS
        (B) LOCATION: 40..765

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    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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                                            Met Lys Ala Thr Leu
55                                           1               5

GTT CTC GGC TCC CTC ATT GTA GGC GCC GTT TCC GCG TAC AAG GCC ACC      102
Val Leu Gly Ser Leu Ile Val Gly Ala Val Ser Ala Tyr Lys Ala Thr
                10                  15                  20
60
ACC ACG CGC TAC TAC GAT GGG CAG GAG GGT GCT TGC GGA TGC GGC TCG     150
Thr Thr Arg Tyr Tyr Asp Gly Gln Glu Gly Ala Cys Gly Cys Gly Ser
            25                  30                  35

65  AGC TCC GGC GCA TTC CCG TGG CAG CTC GGC ATC GGC AAC GGA GTC TAC    198
    Ser Ser Gly Ala Phe Pro Trp Gln Leu Gly Ile Gly Asn Gly Val Tyr
                40                  45                  50
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ACG GCT GCC GGC TCC CAG GCT CTC TTC GAC ACG GCC GGA GCT TCA TGG          246
Thr Ala Ala Gly Ser Gln Ala Leu Phe Asp Thr Ala Gly Ala Ser Trp
        55                  60                  65

TGC GGC GCC GGC TGC GGT AAA TGC TAC CAG CTC ACC TCG ACG GGC CAG          294
Cys Gly Ala Gly Cys Gly Lys Cys Tyr Gln Leu Thr Ser Thr Gly Gln
    70              75                  80                      85

GCG CCC TGC TCC AGC TGC GGC ACG GGC GGT GCT GCT GGC CAG AGC ATC          342
Ala Pro Cys Ser Ser Cys Gly Thr Gly Gly Ala Ala Gly Gln Ser Ile
                90                  95                  100

ATC GTC ATG GTG ACC AAC CTG TGC CCG AAC AAT GGG AAC GCG CAG TGG          390
Ile Val Met Val Thr Asn Leu Cys Pro Asn Asn Gly Asn Ala Gln Trp
            105                 110                 115

TGC CCG GTG GTC GGC GGC ACC AAC CAA TAC GGC TAC AGC TAC CAT TTC          438
Cys Pro Val Val Gly Gly Thr Asn Gln Tyr Gly Tyr Ser Tyr His Phe
            120                 125                 130

GAC ATC ATG GCG CAG AAC GAG ATC TTT GGA GAC AAT GTC GTC GTC GAC          486
Asp Ile Met Ala Gln Asn Glu Ile Phe Gly Asp Asn Val Val Val Asp
            135                 140                 145

TTT GAG CCC ATT GCT TGC CCC GGG CAG GCT GCC TCT GAC TGG GGG ACG          534
Phe Glu Pro Ile Ala Cys Pro Gly Gln Ala Ala Ser Asp Trp Gly Thr
150                 155                 160                 165

TGC CTC TGC GTG GGA CAG CAA GAG ACG GAT CCC ACG CCC GTC CTC GGC          582
Cys Leu Cys Val Gly Gln Gln Glu Thr Asp Pro Thr Pro Val Leu Gly
            170                 175                 180

AAC GAC ACG GGC TCA ACT CCT CCC GGG AGC TCG CCG CCA GCG ACA TCG          630
Asn Asp Thr Gly Ser Thr Pro Pro Gly Ser Ser Pro Pro Ala Thr Ser
            185                 190                 195

TCG AGT CCG CCG TCT GGC GGC GGC CAG CAG ACG CTC TAT GGC CAG TGT          678
Ser Ser Pro Pro Ser Gly Gly Gly Gln Gln Thr Leu Tyr Gly Gln Cys
            200                 205                 210

GGA GGT GCC GGC TGG ACG GGA CCT ACG ACG TGC CAG GCC CCA GGG ACC          726
Gly Gly Ala Gly Trp Thr Gly Pro Thr Thr Cys Gln Ala Pro Gly Thr
            215                 220                 225

TGC AAG GTT CAG AAC CAG TGG TAC TCC CAG TGT CTT CCT TGAGAAGGCC          775
Cys Lys Val Gln Asn Gln Trp Tyr Ser Gln Cys Leu Pro
230                 235                 240

CAAGATAGCC ATGTCTCTCT AGCATTCTTC CGGCGTCAGT CTGATCTGCC TATTTAATCA       835

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(2) INFORMATION FOR SEQ ID NO: 2

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            (A) LENGTH: 166 amino acids
            (B) TYPE: amino acid
            (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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          Ala Tyr Lys Ala Thr Thr Thr Arg Tyr Tyr Asp Gly Gln Glu Gly Ala
                   20                  25              30
 5        Cys Gly Cys Gly Ser Ser Ser Gly Ala Phe Pro Trp Gln Leu Gly Ile
                   35                  40              45
          Gly Asn Gly Val Tyr Thr Ala Ala Gly Ser Gln Ala Leu Phe Asp Thr
               50                  55              60
10        Ala Gly Ala Ser Trp Cys Gly Ala Gly Cys Gly Lys Cys Tyr Gln Leu
          65                  70              75                      80
          Thr Ser Thr Gly Gln Ala Pro Cys Ser Ser Cys Gly Thr Gly Gly Ala
                       85              90                  95
15        Ala Gly Gln Ser Ile Ile Val Met Val Thr Asn Leu Cys Pro Asn Asn
                   100             105             110
          Gly Asn Ala Gln Trp Cys Pro Val Val Gly Gly Thr Asn Gln Tyr Gly
20            115             120             125
          Tyr Ser Tyr His Phe Asp Ile Met Ala Gln Asn Glu Ile Phe Gly Asp
              130             135             140
25        Asn Val Val Val Asp Phe Glu Pro Ile Ala Cys Pro Gly Gln Ala Ala
          145             150             155             160
          Ser Asp Trp Gly Thr Cys Leu Cys Val Gly Gln Gln Glu Thr Asp Pro
                       165             170             175
30        Thr Pro Val Leu Gly Asn Asp Thr Gly Ser Thr Pro Pro Gly Ser Ser
                   180             185             190
          Pro Pro Ala Thr Ser Ser Ser Pro Pro Ser Gly Gly Gly Gln Gln Thr
35            195             200             205
          Leu Tyr Gly Gln Cys Gly Gly Ala Gly Trp Thr Gly Pro Thr Thr Cys
              210             215             220
40        Gln Ala Pro Gly Thr Cys Lys Val Gln Asn Gln Trp Tyr Ser Gln Cys
          225             230             235             240
          Leu Pro
45
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(2) INFORMATION FOR SEQ ID NO: 3:

        (i) SEQUENCE CHARACTERISTICS:
50   (A) LENGTH: 166 amino acids
                (B) TYPE: amino acid
                (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: protein
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        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

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          1                  5              10              15
60        Gly Cys Gly Ser Ser Ser Gly Ala Phe Pro Trp Gln Leu Gly Ile Gly
                   20                  25              30
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Asn Gly Val Tyr Thr Ala Ala Gly Ser Gln Ala Leu Phe Asp Thr Ala
        35                  40              45
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Gly Ala Ser Trp Cys Gly Ala Gly Cys Gly Lys Cys Tyr Gln Leu Thr
        50              55              60
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Ser Thr Gly Gln Ala Pro Cys Ser Ser Cys Gly Thr Gly Gly Ala Ala
    65              70              75                  80
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Gly Gln Ser Ile Ile Val Met Val Thr Asn Leu Cys Pro Asn Asn Gly
                85              90              95
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Asn Ala Gln Trp Cys Pro Val Val Gly Gly Thr Asn Gln Tyr Gly Tyr
            100             105             110
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```
Ser Tyr His Phe Asp Ile Met Ala Gln Asn Glu Ile Phe Gly Asp Asn
        115             120             125
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Val Val Val Asp Phe Glu Pro Ile Ala Cys Pro Gly Gln Ala Ala Ser
    130             135             140
```

```
Asp Trp Gly Thr Cys Leu Cys Val Gly Gln Gln Glu Thr Asp Pro Thr
145             150             155             160
```

25
```
Pro Val Leu Gly Asn Asp
            165
```

30
(2) INFORMATION FOR SEQ ID NO: 4:

    (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 2211 base pairs
      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
35
      (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

```
GAATTCTCAC GGTGAATGTA GGCCTTTTGT AGGGTAGGAA TTGTCACTCA AGCACCCCCA      60
40
ACCTCCATTA CGCCTCCCCC ATAGAGTTCC CAATCAGTGA GTCATGGCAC TGTTCTCAAA     120

TAGATTGGGG AGAAGTTGAC TTCCGCCCAG AGCTGAAGGT CGCACAACCG CATGATATAG     180

45  GGTCGGCAAC GGCAAAAAAG CACGTGGCTC ACCGAAAAGC AAGATGTTTG CGATCTAACA     240

TCCAGGAACC TGGATACATC CATCATCACG CACGACCACT TTGATCTGCT GGTAAACTCG     300

TATTCGCCCT AAACCGAAGT GCGTGGTAAA TCTACACGTG GGCCCCTTTC GGTATACTGC     360
50
GTGTGTCTTC TCTAGGTGCA TTCTTTCCTT CCTCTAGTGT TGAATTGTTT GTGTTGGGAG     420

TCCGAGCTGT AACTACCTCT GAATCTCTGG AGAATGGTGG ACTAACGACT ACCGTGCACC     480

55  TGCATCATGT ATATAATAGT GATCCTGAGA AGGGGGGTTT GGAGCAATGT GGGACTTTGA     540

TGGTCATCAA ACAAAGAACG AAGACGCCTC TTTTGCAAAG TTTTGTTTCG GCTACGGTGA     600

AGAACTGGAT ACTTGTTGTG TCTTCTGTGT ATTTTTGTGG CAACAAGAGG CCAGAGACAA     660
60
TCTATTCAAA CACCAAGCTT GCTCTTTTGA GCTACAAGAA CCTGTGGGGT ATATATCTAG     720

AGTTGTGAAG TCGGTAATCC CGCTGTATAG TAATACGAGT CGCATCTAAA TACTCCGAAG     780

65  CTGCTGCGAA CCCGGAGAAT CGAGATGTGC TGGAAAGCTT CTAGCGAGCG GCTAAATTAG     840

CATGAAAGGC TATGAGAAAT TCTGGAGACG GCTTGTTGAA TCATGGCGTT CCATTCTTCG     900
```

```
ACAAGCAAAG CGTTCCGTCG CAGTAGCAGG CACTCATTCC CGAAAAAACT CGGAGATTCC    960

TAAGTAGCGA TGGAACCGGA ATAATATAAT AGGCAATACA TTGAGTTGCC TCGACGGTTG   1020

CAATGCAGGG GTACTGAGCT TGGACATAAC TGTTCCGTAC CCCACCTCTT CTCAACCTTT   1080

GGCGTTTCCC TGATTCAGCG TACCCGTACA AGTCGTAATC ACTATTAACC CAGACTGACC   1140

GGACGTGTTT TGCCCTTCAT TTGGAGAAAT AATGTCATTG CGATGTGTAA TTTGCCTGCT   1200

TGACCGACTG GGGCTGTTCG AAGCCCGAAT GTAGGATTGT TATCCGAACT CTGCTCGTAG   1260

AGGCATGTTG TGAATCTGTG TCGGGCAGGA CACGCCTCGA AGGTTCACGG CAAGGGAAAC   1320

CACCGATAGC AGTGTCTAGT AGCAACCTGT AAAGCCGCAA TGCAGCATCA CTGGAAAATA   1380

CAAACCAATG GCTAAAAGTA CATAAGTTAA TGCCTAAAGA AGTCATATAC CAGCGGCTAA   1440

TAATTGTACA ATCAAGTGGC TAAACGTACC GTAATTTGCC AACGCGTTGT GGGGTTGCAG   1500

AAGCAACGGC AAAGCCCACT TCCCACGTTT GTTTCTTCAC TCAGTCCAAT CTCAGCTGGT   1560

GATCCCCCAA TTGGGTCGCT TGTTTGTTCC GGTGAAGTGA AAGAAGACAG AGGTAAGAAT   1620

GTCTGACTCG GAGCGTTTTG CATACAACCA AGGGCAGTGA TGGAAGACAG TGAAATGTTG   1680

ACATTCAAGG AGTATTTAGC CAAGGGATGCTTGAGTGTATC GTGTAAGGAG GTTTGTCTGC   1740

CGATACGACG AATACTGTAT AGTCACTTCT GATGAAGTGG TCCATATTGA AATGTAAGTC   1800

GGCACTGAAC AGGCAAAAGA TTGAGTTGAA ACTGCCTAAG ATCTCGGGCC CTCGGGCTTC   1860

GGCTTTGGGT GTACATGTTT GTGCTCCGGG CAAATGCAAA GTGTGGTAGG ATCGACACAC   1920

TGCTGCCTTT ACCAAGCAGC TGAGGGTATG TGATAGGCAA ATGTTCAGGG GCCACTGCAT   1980

GGTTTCGAAT AGAAAGAGAA GCTTAGCCAA GAACAATAGC CGATAAAGAT AGCCTCATTA   2040

AACGAAATGA GCTAGTAGGC AAAGTCAGCG AATGTGTATA TATAAAGGTT CGAGGTCCGT   2100

GCCTCCCTCA TGCTCTCCCC ATCTACTCAT CAACTCAGAT CCTCCAGGAG ACTTGTACAC   2160

CATCTTTTGA GGCACAGAAA CCCAATAGTC AACCGCGGAC TGCGCATCAT G            2211
```

(2) INFORMATION FOR SEQ ID NO: 5:

　　　　(i) SEQUENCE CHARACTERISTICS:
　　　　　　(A) LENGTH: 1627 base pairs
　　　　　　(B) TYPE: nucleic acid
　　　　　　(C) STRANDEDNESS: single
　　　　　　(D) TOPOLOGY: linear

　　　　(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

```
GGCGGTATTG GCTACAGCGG CCCCACGGTC TGCGCCAGCG GCACAACTTG CCAGGTCCTG     60

AACCCTTACT ACTCTCAGTG CCTGTAAAGC TCCGTGCGAA AGCCTGACGC ACCGGTAGAT    120

TCTTGGTGAG CCCGTATCAT GACGGCGGCG GGAGCTACAT GGCCCGGGT GATTTATTTT    180

TTTTGTATCT ACTTCTGACC CTTTTCAAAT ATACGGTCAA CTCATCTTTC ACTGGAGATG    240

CGGCCTGCTT GGTATTGCGA TGTTGTCAGC TTGGCAAATT GTGGCTTTCG AAAACACAAA    300

ACGATTCCTT AGTAGCCATG CATCGGGATC CTTTAAGATA ACGGAATAGA AGAAAGAGGA    360
```

```
     AATTAAAAAA AAAAA AAAA CAAACATCCC GTTCATAACC CGTAGAATCG CCGCTCTTCG .   420

     TGTATCCCAG TACCACGGCA AAGGTATTTC ATGATCGTTC AATGTTGATA TTGTTCCCGC    480

5    CAGTATGGCT GCACCCCCAT CTCCGCGAAT CTCCTCTTCT CGAACGCGGT AGTGGCGCGC    540

     CAATTGGTAA TGACCATAGG GAGACAAACA GCATAATAGC AACAGTGGAA ATTAGTGGCG    600

     CAATAATTGA GAACACAGTG AGACCATAGC TGGCGGCCTG GAAAGCACTG TTGGAGACCA    660

10   ACTTGTCCGT TGCGAGGCCA ACTTGCATTG CTGTCAAGAC GATGACAACG TAGCCGAGGA    720

     CCGTCACAAG GGACGCAAAG TTGTCGCGGA TGAGGTCTCC GTAGATGGCA TAGCCGGCAA    780

15   TCCGAGAGTA GCCTCTCAAC AGGTGGCCTT TTCGAAACCG GTAAACCTTG TTCAGACGTC    840

     CTAGCCGCAG CTCACCGTAC CAGTATCGAG GATTGACGGC AGAATAGCAG TGGCTCTCCA    900

     GGATTTGACT GGACAAAATC TTCCAGTATT CCCAGGTcAC AGTGTCTGGC AGAAGTCCCT    960

20   TCTCGCGTGC ANTCGAAAGT CGCTATAGTG CGCAATGAGA GCACAGTAGG AGAATAGGAA   1020

     CCCGCGAGCA CATTGTTCAA TCTCCACATG AATTGGATGA CTGCTGGGCA GAATGTGCTG   1080

25   CCTCCAAAAT CCTGCGTCCA ACAGATACTC TGGCAGGGGC TTCAGATGAA TGCCTCTGGG   1140

     CCCCCAGATA AGATGCAGCT CTGGATTCTC GGTTACNATG ATATCGCGAG AGAGCACGAG   1200

     TTGGTGATGG AGGGACAGGA GGCATAGGTC GCGCAGGCCC ATAACCAGTC TTGCACAGCA   1260

30   TTGATCTTAC CTCACGAGGA GCTCCTGATG CAGAAACTCC TCCATGTTGC TGATTGGGTT   1320

     GAGAATTTCA TCGCTCCTGG ATCGTATGGT TGCTGGCAAG ACCCTGCTTA ACCGTGCCGT   1380

35   GTCATGGTCA TCTCTGGTGG CTTCGTCGCT GGCCTGTCTT TGCAATTCGA CAGCAAATGG   1440

     TGGAGATCTC TCTATCGTGA CAGTCATGGT AGCGATAGCT AGGTGTCGTT GCACGCACAT   1500

     AGGCCGAAAT GCGAAGTGGA AAGAATTTCC CGGNTGCGGA ATGAAGTCTC GTCATTTTGT   1560

40   ACTCGTACTC GACACCTCCA CCGAAGTGTT AATAATGGAT CCACGATGCC AAAAAGCTTG   1620
                                                                         1627
     TGCATGC
```

45   (2) INFORMATION FOR SEQ ID NO: 6:

          (i) SEQUENCE CHARACTERISTICS:
              (A) LENGTH: 1137 base pairs
50   (B) TYPE: nucleic acid
              (C) STRANDEDNESS: single
              (D) TOPOLOGY: linear

          (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

```
55   GAATTCTCAC GGTGAATGTA GGCCTTTTGT AGGGTAGGAA TTGTCACTCA AGCACCCCCA     60

     ACCTCCATTA CGCCTCCCCC ATAGAGTTCC CAATCAGTGA GTCATGGCAC TGTTCTCAAA    120

     TAGATTGGGG AGAAGTTGAC TTCCGCCCAG AGCTGAAGGT CGCACAACCG CATGATATAG    180

60   GGTCGGCAAC GGCAAAAAAG CACGTGGCTC ACCGAAAAGC AAGATGTTTG CGATCTAACA    240

     TCCAGGAACC TGGATACATC CATCATCACG CACGACCACT TTGATCTGCT GGTAAACTCG    300

65   TATTCGCCCT AAACCGAAGT GCGTGGTAAA TCTACACGTG GGCCCCTTTC GGTATACTGC    360
```

```
GTGTGTCTTC TCTAGGTGCA TTCTTTCCTT CCTCTAGTGT TGAATTGTTT GTGTTGGGAG    420

TCCGAGCTGT AACTACCTCT GAATCTCTGG AGAATGGTGG ACTAACGACT ACCGTGCACC    480

TGCATCATGT ATATAATAGT GATCCTGAGA AGGGGGGTTT GGAGCAATGT GGGACTTTGA    540

TGGTCATCAA ACAAAGAACG AAGACGCCTC TTTTGCAAAG TTTTGTTTCG GCTACGGTGA    600

AGAACTGGAT ACTTGTTGTG TCTTCTGTGT ATTTTTGTGG CAACAAGAGG CCAGAGACAA    660

TCTATTCAAA CACCAAGCTT GCTCTTTTGA GCTACAAGAA CCTGTGGGGT ATATATCTAG    720

TGGCCAGAAT GCCTAGGTCA CCTCTAGAGA GTTGAAACTG CCTAAGATCT CGGGCCCTCG    780

GGCTTCGGCT TTGGGTGTAC ATGTTTGTGC TCCGGGCAAA TGCAAAGTGT GGTAGGATCG    840

ACACACTGCT GCCTTTACCA AGCAGCTGAG GGTATGTGAT AGGCAAATGT TCAGGGGCCA    900

CTGCATGGTT TCGAATAGAA AGAGAAGCTT AGCCAAGAAC AATAGCCGAT AAAGATAGCC    960

TCATTAAACG AAATGAGCTA GTAGGCAAAG TCAGCGAATG TGTATATATA AAGGTTCGAG   1020

GTCCGTGCCT CCCTCATGCT CTCCCCATCT ACTCATCAAC TCAGATCCTC CAGGAGACTT   1080

GTACACCATC TTTTGAGGCA CAGAAACCCA ATAGTCAACC GCGGACTGCG CATCATG      1137
```

(2) INFORMATION FOR SEQ ID NO: 7:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 24 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

```
GGTCTGAAGG ACGTGGAATG ATGG                                            24
```

(2) INFORMATION FOR SEQ ID NO: 8:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 51 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

```
GATGCATCGA TCGTCCGCGG GTTGAGAGAA GTTGTTGGAT TGATCAAAAA G             51
```

(2) INFORMATION FOR SEQ ID NO: 9:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 42 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

```
GAGAGACCGC GGTGATCTTC CATCTCGTGT CTTGCTTGTA AC                       42
```

(2) INFORMATION FOR SEQ ID NO: 10:

    (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 30 base pairs
5   (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

10

ATCGTGGATC CATTATTAAC ACTTCGGTGG   30

48

Claims:

1. An isolated DNA sequence, c h a r a c t e r i z e d in that it codes for a *Trichoderma* enzyme having endoglucanase activity, the molecular weight of the unglycosylated form of said enzyme being 20 to 25 kDa and said enzyme comprising a core domain, a linker region and a cellulose binding domain, and functional parts thereof.

2. The DNA sequence according to claim 1, wherein the DNA sequence hybridizes to the DNA sequence of SEQ ID NO. 1 or to the DNA sequence of SEQ ID NO. 11.

3. The DNA sequence according to claim 1, wherein the DNA sequence codes for the amino acid sequence of SEQ ID NO. 2.

4. The DNA sequence according to claim 1, wherein the DNA sequence is the DNA sequence of SEQ ID NO. 1 or to the DNA sequence of SEQ ID NO. 11.

5. A DNA sequence, which codes for a polypeptide having endoglucanase activity, said sequence coding for the sequence of SEQ ID NO. 3 or functional equivalents thereof.

6. A vector construction, c h a r a c t e r i z e d in that it comprises the DNA sequence of any one of claims 1 to 5.

7. A microorganism host, c h a r a c t e r i z e d in that it has been transformed with the DNA sequence of any one of claims 1 to 5 or with a vector construction of claim 6 and is able to express said DNA sequence.

8. The host according to claim 7, wherein said host is a fungal or yeast host.

9. The host according to claims 7 or 8, wherein said host is *Trichoderma*.

10. The host according to claims 7 or 8, wherein said host is *Saccharomyces*.

11. A culture medium, c h a r a c t e r i z e d in that is comprises the enzymes secreted from the host of claims 7 to 10.

12. A product derived from the culture medium of claim 11 by purifying, concentrating, drying or immobilizing said culture medium.

13. A method for isolating a DNA sequence coding for *Trichoderma* enzyme having endoglucanase activity,

c h a r a c t e r i z e d by

- enriching the mRNA pool of a *Trichoderma* strain producing endoglucanase activity in respect of the mRNA of the endoglucanase by culturing the *Trichoderma* strain in conditions which will induce the endoglucanase production of said strain,
- isolating mRNA from the strain,
- preparing cDNA corresponding to the isolated mRNA,
- placing the cDNA thus obtained in a vector under the control of a yeast promoter,
- transforming the recombinant plasmids into a yeast strain which naturally does not produce the endoglucanase in order to provide an expression library,
- cultivating the yeast clones thus obtained on a culture medium in order to express the expression library in the yeast,
- separating the yeast clones producing the endoglucanase from the other yeast clones,
- isolating the plasmid-DNA of said separated yeast clones, and,
- if desired, sequencing the DNA in order to determine the DNA sequence coding for the endoglucanase.

14. The method according to claim 13, wherein the recombinant plasmids are transformed into a strain of the yeast *Saccharomyces cerevisiae*.

15. The method according to claim 13, wherein the yeast clones are cultivated on a culture medium containing at least one substrate selected from the group comprising β-glucan, hydroxyethyl cellulose, methylumbelliferyl lactoside and methylumbelliferyl cellobioside.

16. A method for constructing a *Trichoderma* host capable of expressing an endoglucanase

50

enzyme, c h a r a c t e r i z e d in that it comprises

    a)   isolating the DNA sequence coding for an endoglucanase, the molecular weight of which in unglycosylated form is 20 to 25 kDa, or parts thereof, from a suitable donor strain,

    b)   constructing a vector carrying said DNA sequence and

    c)   transforming the vector obtained into a *Trichoderma* host.

17. A method for producing a *Trichoderma* endoglucanase enzyme, c h a r a c t e r i z e d in that it comprises the steps of

    a)   isolating the DNA sequence coding for an endoglucanase, the molecular weight of which in unglycosylated form is 20 to 25 kDa, or functional parts thereof, from a suitable donor strain

    b)   constructing a vector carrying said DNA sequence,

    c)   transforming the vector obtained to a *Trichoderma* host to obtain a recombinant host strain,

    d)   cultivating said recombinant host strain under conditions permitting expression of said endoglucanase, and

    e)   recovering said endoglucanase.

18. A method for constructing a *Saccharomyces* host capable of expressing an endoglucanase enzyme, c h a r a c t e r i z e d in that it comprises

    a)   isolating the DNA sequence coding for an endoglucanase, the molecular weight of whose unglycosylated form being 20 to 25 kDa, and functional parts thereof, from a suitable donor strain,

    b)   constructing a vector carrying said DNA sequence and

    c)   transforming the vector obtained into a *Saccharomyces* host.

19. A method for producing a *Trichoderma* endoglucanase enzyme, c h a r a c t e r i z e d by

    a)   isolating the DNA sequence coding for an endoglucanase, the molecular weight of which is 20 to 25 kDa in unglycosylated form, or functional parts thereof, from a suitable donor strain

b)   constructing a vector carrying said DNA sequence,

c)   transforming the vector obtained to a *Saccharomyces* host to obtain a recombinant host strain,

d)   cultivating said recombinant host strain under conditions permitting expression of said endoglucanase, and

e)   recovering said endoglucanase.

20. An enzyme preparation, c h a r a c t e r i z e d in that it contains an endoglucanase enzyme having the amino acid sequence of SEQ ID NO. 2 or functional derivatives thereof.

21. An enzyme preparation, c h a r a c t e r i z e d in that it contains elevated levels of an endoglucanase enzyme having in unglycosylated form a molecular weight in the range from 20 to 25 kDa, or functional parts thereof, and exhibiting catalytic activity towards the substrates β-glucan.

22. The enzyme preparation according to claim 22, wherein the endoglucanase enzymes exhibits activity towards crystalline cellulose substrate.

23. A method for enzymatically modifying a cellulosic substrate, c h a r a c t e r - i z e d by contacting said substrate with an enzyme preparation according to any one of claims 20 to 22.

24. The method according to claim 23, wherein the cellulosic substrate is fibrous.

```
GATCTTCCATCTCGTGTCTTGCTTGTAACCATCGTGACCATGAAGGCAACTCTGGTTCTC    60
                                        MetLysAlaThrLeuValLeu     7

GGCTCCCTCATTGTAGGCGCCGTTTCCGCGTACAAGGCCACCACCACGCGCTACTACGAT   120
GlySerLeuIleValGlyAlaValSerAlaTyrLysAlaThrThrThrArgTyrTyrAsp    27

GGGCAGGAGGGTGCTTGCGGATGCGGCTCGAGCTCCGGCGCATTCCCGTGGCAGCTCGGC   180
GlyGlnGluGlyAlaCysGlyCysGlySerSerSerGlyAlaPheProTrpGlnLeuGly    47

ATCGGCAACGGAGTCTACACGGCTGCCGGCTCCCAGGCTCTCTTCGACACGGCCGGAGCT   240
IleGlyAsnGlyValTyrThrAlaAlaGlySerGlnAlaLeuPheAspThrAlaGlyAla    67

TCATGGTGCGGCGCCGGCTGCGGTAAATGCTACCAGCTCACCTCGACGGGCCAGGCGCCC   300
SerTrpCysGlyAlaGlyCysGlyLysCysTyrGlnLeuThrSerThrGlyGlnAlaPro    87

TGCTCCAGCTGCGGCACGGGCGGTGCTGCTGGCCAGAGCATCATCGTCATGGTGACCAAC   360
CysSerSerCysGlyThrGlyGlyAlaAlaGlyGlnSerIleIleValMetValThrAsn   107

CTGTGCCCGAACAATGGGAACGCGCAGTGGTGCCCGGTGGTCGGCGGCACCAACCAATAC   420
LeuCysProAsnAsnGlyAsnAlaGlnTrpCysProValValGlyGlyThrAsnGlnTyr   127

GGCTACAGCTACCATTTCGACATCATGGCGCAGAACGAGATCTTTGGAGACAATGTCGTC   480
GlyTyrSerTyrHisPheAspIleMetAlaGlnAsnGluIlePheGlyAspAsnValVal   147

GTCGACTTTGAGCCCATTGCTTGCCCCGGGCAGGCTGCCTCTGACTGGGGGACGTGCCTC   540
ValAspPheGluProIleAlaCysProGlyGlnAlaAlaSerAspTrpGlyThrCysLeu   167

TGCGTGGGACAGCAAGAGACGGATCCCACGCCCGTCCTCGGCAACGACACGGGCTCAACT   600
CysValGlyGlnGlnGluThrAspProThrProValLeuGlyAsnAspThrGlySerThr   187
                                                   *   ↳ B

CCTCCCGGGAGCTCGCCGCCAGCGACATCGTCGAGTCCGCCGTCTGGCGGCGGCCAGCAG   660
ProProGlySerSerProProAlaThrSerSerSerProProSerGlyGlyGlyGlnGln   207
                                                   B ↰→ A

ACGCTCTATGGCCAGTGTGGAGGTGCCGGCTGGACGGGACCTACGACGTGCCAGGCCCCA   720
ThrLeuTyrGlyGlnCysGlyGlyAlaGlyTrpThrGlyProThrThrCysGlnAlaPro   227

GGGACCTGCAAGGTTCAGAACCAGTGGTACTCCCAGTGTCTTCCTTGAGAAGGCCCAAGA   780
GlyThrCysLysValGlnAsnGlnTrpTyrSerGlnCysLeuPro                  242

TAGCCATGTCTCTCTAGCATTCTTCCGGCGTCAGTCTGATCTGCCTATTTAATCAGGTCA   840

GTCAATATGTATCCAGAGATAATAAATTATGTATATTATAGCAG(A)₃₉              923
```

FIG. 1

```
        206
EGV    QQTLYGQCGGAGWTGPTTCQAEG-TCKVQNQNYSQCLP***
       405
EGI    TQTHWGQCGGIGYSGCKTC-TSGTTCQYSNDYYSQCL***
       442
CBHI   TQSHYGQCGGIGYSGPTVC-ASGTTCQVLNPYYSQCL***
       1
EGII   QQTVWGQCGGIGWSGPTNC-APGSACSTLNPYYAQCI
       3
CBHII  CSSVWGQCGGQNWEGPTCC-ASGSTCVYSNDYYSQCL
```

FIG. 2A

```
        104
EGV    ----TGSTPPGSSPP--A-TSSS---PPSGGG
       380
EGI    PPPASSTTF-STTRR-S--STTS-S-SPSCTQT
       438
CBHI   PGGNRGTT---TTRRP-A--TTTGS-SPGPTQS
       37
EGII   PGATTITT--S-TRPPSGPTTTTRATS-TSSSTPPTSS
       39
CBHII  PGAASSSS--S-TRAAS--TTSRV-SPTTS
       61
       RSSS---ATPPP-G-STTTRV-PPVGSGTATYS
```

FIG. 2B

BamHI
BglII   XhoI
XhoI   BglII
EcoRI
BglII   egl 5   PGKt
PGKp   URA3

pAS4
6.5 kb

BamHI

2µ   BglII
ClaI

ClaI

FIG. 3

SalI
XhoI SacI   BamHI
BamHI   SacI   XhoI
XbaI   KpnI
BstEI   SacI
BsmI   egl 5   NsiI
BalI   BamHI

cbh1p   cbh1t

pAS16
6 kb

EcoRI
SalI   BamHI
EcoRI   Amp   SphI
Ncol

FIG. 4

SUBSTITUTE SHEET

(XbaI) BalI BsmI   BstEII XbaI KpnI PstI XhoI (XbaI)
5' CTAGTGGCCAGAATGCCTAGGTCACCTCTAGAGGTACCCTGCAGCTCGAG
        ACCGGTCTTACGGATCCAGTGGAGATCTCCATGGGACGTCGAGCTCGATC

5' GGACTGCGCATCATGCAG
   CGCCTGACGCGTAGTACGTCCTAG
   KspI┘   └BamHI

SalI
5' AATTCGTCGACG
     GCAGCTGCTTAA
   EcoRI┘   └EcoRI

pMLO16
9.3 kb

cbh1-promoter

amp^r

ORI

lacZ

cbh1-terminator

NcoI
SphI
BamHI

BamHI
NaeI

EcoRI

lacZ   cbh1
5'..TAATAATAACCGGCGGTATTGG

FIG. 5

FIG. 6

FIG. 7A
SUBSTITUTE SHEET

FIG. 7B
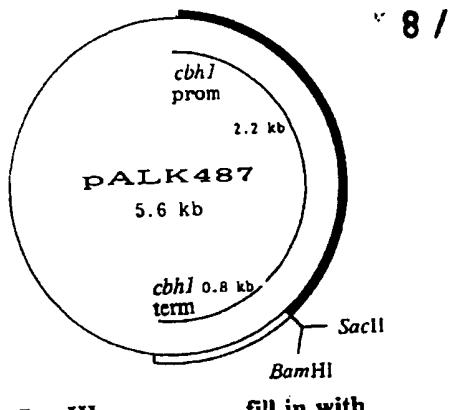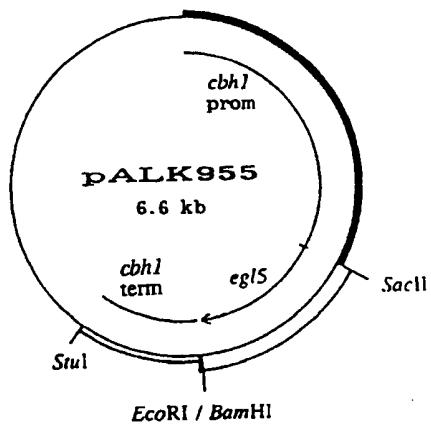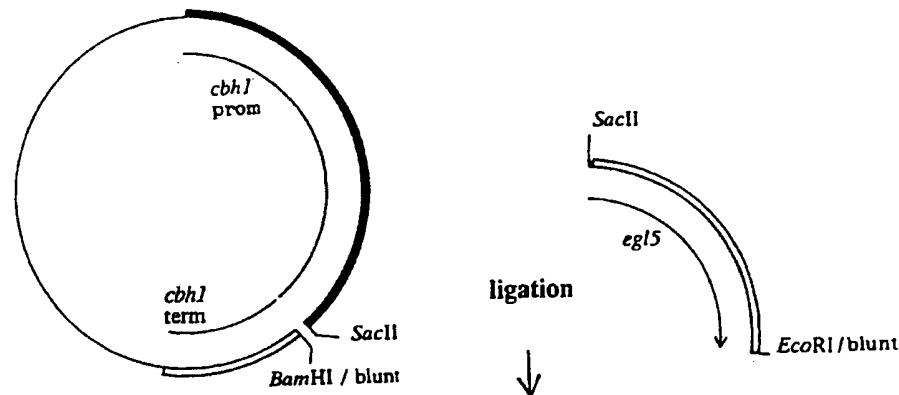
FIG. 7C

FIG. 7D

FIG. 8

pH-Stability
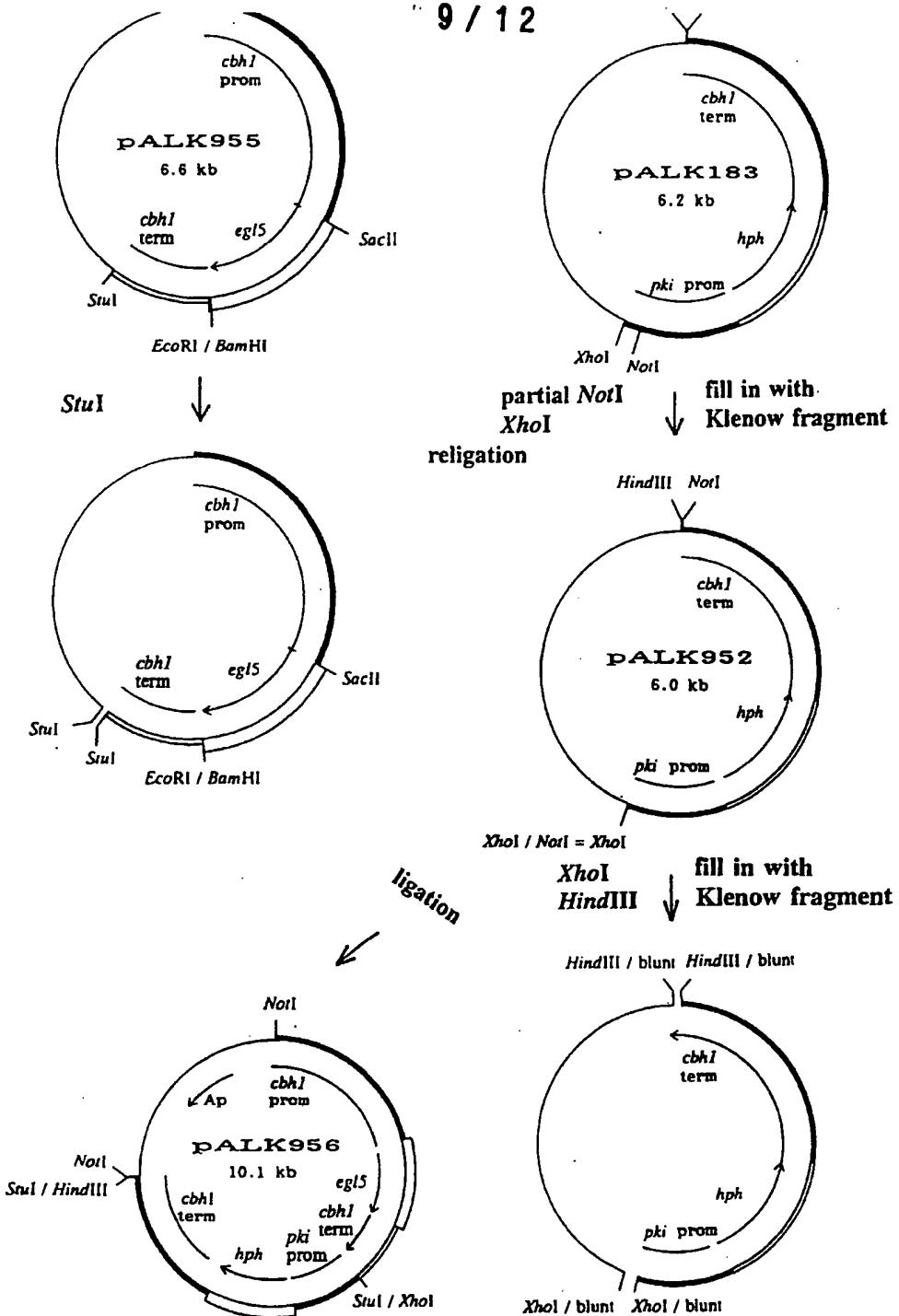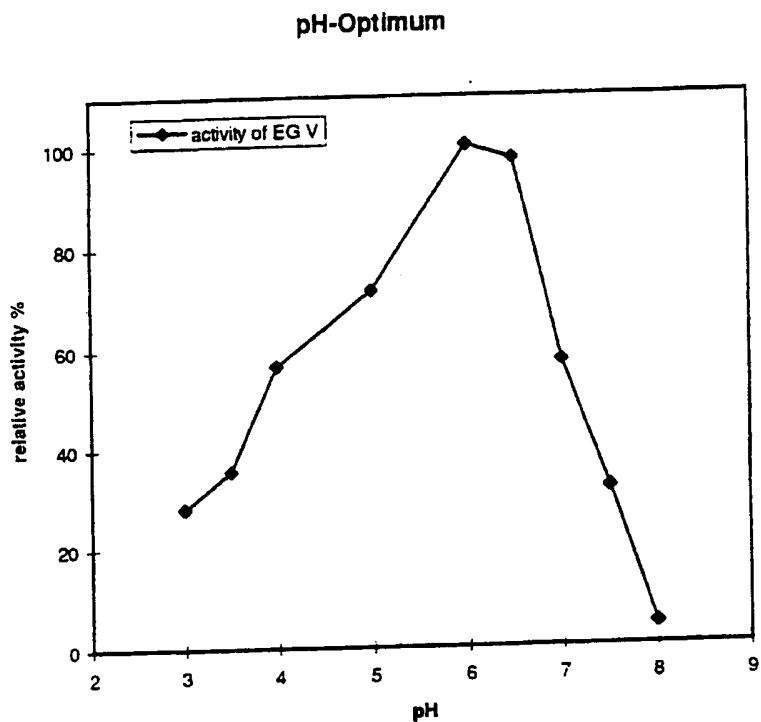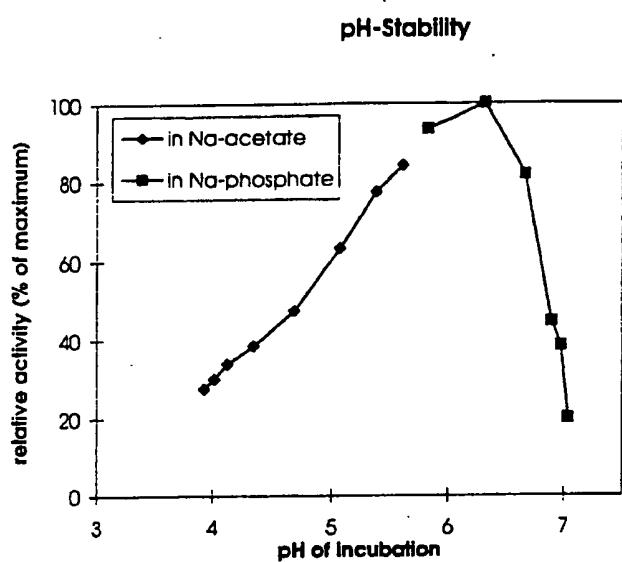

FIG. 9

```
GATCTTCCATCTCGTGTCTTGCTTGTAACCATCGTGACCATGAAGGCAACTCTGGTTCTC        60
                                        MetLysAlaThrLeuValLeu         7

GGCTCCCTCATTGTAGGCGCCGTTTCCGCGTACAAGGCCACCACCACGGCAAGTCTACAT       120
GlySerLeuIleValGlyAlaValSerAlaTyrLysAlaThrThrThr                    23

GCTTCCAGGTCACAACGTCTGCTCAACAACCTCTAACCGAAAGGCCAGCGCTACTACGAT       180
         intron 1 (60 bp)                            ArgTyrTyrAsp    27

GGGCAGGAGGGTGCTTGCGGATGCGGCTCGAGCTCCGGCGCATTCCCGTGGCAGGTAAAC       240
GlyGlnGluGlyAlaCysGlyCysGlySerSerSerGlyAlaPheProTrpGln              45

ATTCGACCTTGTCTGGGGCAGGGACTCGTCACTTACATCCTCTCTCTCTCTGCAGCTCG       300
        intron 2 (62 bp)                                  LeuG      46

GCATCGGCAACGGAGTCTACACGGCTGCCGGCTCCCAGGCTCTCTTCGACACGGCCGGAG       360
lyIleGlyAsnGlyValTyrThrAlaAlaGlySerGlnAlaLeuPheAspThrAlaGlyA        66

CTTCATGGTGCGGCGCCGGCTGCGGTAAATGCTACCAGCTCACCTCGACGGGCCAGGCGC       420
laSerTrpCysGlyAlaGlyCysGlyLysCysTyrGlnLeuThrSerThrGlyGlnAlaP        86

CCTGCTCCAGCTGCGGCACGGGCGGTGCTGCTGGCCAGAGCATCATCGTCATGGTGACCA       480
roCysSerSerCysGlyThrGlyGlyAlaAlaGlyGlnSerIleIleValMetValThrA        106

ACCTGTGCCCGAACAATGGGAACGCGCAGTGGTGCCCGGTGGTCGGCGGCACCAACCAAT       540
snLeuCysProAsnAsnGlyAsnAlaGlnTrpCysProValValGlyGlyThrAsnGlnT        126

ACGGCTACAGCTACCATTTCGACATCATGGCGCAGAACGAGATCTTTGGAGACAATGTCG       600
yrGlyTyrSerTyrHisPheAspIleMetAlaGlnAsnGluIlePheGlyAspAsnValV        146

TCGTCGACTTTGAGCCCATTGCTTGCCCCGGGCAGGCTGCCTCTGACTGGGGGACGTGCC       660
alValAspPheGluProIleAlaCysProGlyGlnAlaAlaSerAspTrpGlyThrCysL        166

TCTGCGTGGGACAGCAAGAGACGGATCCCACGCCCGTCCTCGGCAACGACACGGGCTCAA       720
euCysValGlyGlnGlnGluThrAspProThrProValLeuGlyAsnAspThrGlySerT        186

CTCCTCCCGGGAGCTCGCCGCCAGCGACATCGTCGAGTCCGCCGTCTGGCGGCGGCCAGC       780
hrProProGlySerSerProProAlaThrSerSerSerProProSerGlyGlyGlyGlnG        206

AGACGCTCTATGGCCAGTGTGGAGGTGCCGGCTGGACGGGACCTACGACGTGCCAGGCCC       840
lnThrLeuTyrGlyGlnCysGlyGlyAlaGlyTrpThrGlyProThrThrCysGlnAlaP        226

CAGGGACCTGCAAGGTTCAGAACCAGTGGTACTCCCAGTGTCTTCCTTGAGAAGGCCCAA       900
roGlyThrCysLysValGlnAsnGlnTrpTyrSerGlnCysLeuPro                     242

GATAGCCATGTCTCTCTAGCATTCTTCCGGCGTCAGTCTGATCTGCCTATTTAATCAGGT       960

CAGTCAATATGTATCCAGAGATAATAAATTATGTATATTATAGCAG (A)₃₉               1044
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FIG. 10

INTERNATIONAL SEARCH REPORT

	International application No.
	PCT/FI 94/00234

A. CLASSIFICATION OF SUBJECT MATTER

IPC5: C12N 9/42, C12N 15/56
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC5: C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPODOC, MEDLINE, BIOSIS, STRAND

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	TRENDS IN BIOTECHNOLOGY, Volume 5, Sept 1987, Jonathan Knowles et al, "Cellulase families and their genes", page 255 - page 261, the whole document, especially p. 257, c. 3, line 3-8	1-24
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X	Chemical Abstracts, Volume 109, No 25, 19 December 1988 (19.12.88), (Columbus, Ohio, USA), Voragen, A. G. J. et al, "Cellulase of a mutant strain of Trichoderma viride", THE ABSTRACT No 225362p, Methods Enzymol. 1988, 160, 243-251	1-24
	--	

X Further documents are listed in the continuation of Box C.   X See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	
"E" erlier document but published on or after the international filing date	"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 Sept 1994	20 -09- 1994
Name and mailing address of the ISA/	Authorized officer
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INTERNATIONAL SEARCH REPORT

International application No.

PCT/FI 94/00234

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO, A1, 9206184 (GENENCOR INTERNATIONAL, INC.), 16 April 1992 (16.04.92), see the whole document, especially Table I p. 32, line 21 - p. 33 line 13	1-24

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International application No.

PCT/FI 94/00234

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A1- 9206184	16/04/92	CA-A- 2093423 EP-A- 0586375	01/12/92 16/03/94